(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 February 2001 (15.02.2001)

PCT

(10) International Publication Number
WO 01/11060 A2

(51) International Patent Classification[7]: C12N 15/74, 15/62

(21) International Application Number: PCT/DK00/00437

(22) International Filing Date: 4 August 2000 (04.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 1999 01105 6 August 1999 (06.08.1999) DK

(71) Applicant *(for all designated States except US)*: **BIOTEKNOLOGISK INSTITUT** [DK/DK]; Kogle Allé 2, DK-2970 Hoersholm (DK).

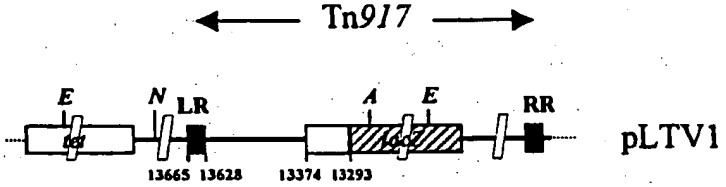
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **RAVN, Peter** [DK/DK]; Hegnsvej 123, DK-2850 Naerum (DK). **MADSEN, Soeren, Michael** [DK/DK]; 1st floor, Baldersgade 11, DK-2200 Copenhagen N (DK). **VRANG, Astrid** [DK/DK]; Langs Hegnet 76, DK-2800 Lyngby (DK). **ISRAELSEN, Hans** [DK/DK]; Proevestensvej 1A, DK-3450 Alleroed (DK). **JOHNSEN, Mads, Groenvold** [DK/DK]; Sveasvej 5, 3rd floor, DK-1917 Frederiksberg C (DK). **BREDMOSE, Lars** [DK/DK]; Brynhildegade 9, 3rd floor, DK-2200 Copenhagen N (DK). **ARNAU, José** [ES/DK]; Melvillevej 9, DK-2900 Hellerup (DK).

(74) Agent: **PLOUGMANN, VINGTOFT & PARTNERS A/S**; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG,

[Continued on next page]

(54) Title: METHOD OF ISOLATING SECRETION SIGNALS IN LACTIC ACID BACTERIA AND NOVEL SECRETION SIGNALS ISOLATED FROM *LACTOCOCCUS LACTIS*



Plasmid	Deleted Region	Sequence Position	Transposition
pPRA5A	———	13647-13374	-
pPRA5B	———	13626-13374	+
pPRA5C	———	13608-13374	+
pPRA5D	———	13589-13374	+

(57) Abstract: A method of identifying nucleotide sequences coding for signal peptides in lactic acid bacteria, using a DNA molecule comprising a transposon including a promoterless reporter gene from which DNA molecule a region between the LR and the reporter gene is deleted and the DNA molecule comprises a DNA sequence coding for a secretion reporter molecule. By deleting the region between the LR and the reporter gene, stop codons in-frame with the secretion reporter molecule is removed which upon transposition permits translational fusions from upstream the LR.

WO 01/11060 A2

MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD OF ISOLATING SECRETION SIGNALS IN LACTIC ACID BACTERIA AND NOVEL SECRETION SIGNALS ISOLATED FROM *LACTOCOCCUS LACTIS*

FIELD OF INVENTION

The present invention relates to the field of microbial expression systems, in particular to means of improving the level of secretion of homologous or heterologous gene products in recombinant host cells. Specifically, there is provided the means of identifying and isolating sequences coding for secretion signals in lactic acid bacteria and novel signal peptides isolated from *Lactococcus* spp., and mutants of such signal peptides having enhanced efficiency.

TECHNICAL BACKGROUND AND PRIOR ART

The group of Gram-positive bacteria that are generally referred to as lactic acid bacteria including *Lactococcus* spp. such as *Lactococcus lactis*, *Lactobacillus* spp., *Streptococcus* spp., *Leuconostoc* spp. and *Oenococcus* spp. are commonly used in the manufacturing of food products and feedstuffs, e.g. as dairy starter cultures in the manufacturing of fermented milk products such as butter, cheese and yoghurt. *Lactococcus lactis* is a typical example of a Gram-positive bacterium used for the manufacturing of a wide range of fermented milk products.

Besides, lactic acid bacteria are currently used as recombinant host cells for the production of heterologous and homologous gene products such as pharmaceutically active products or enzymes. Among the emerging industrial applications for *L. lactis*, recent work by the inventors has focused on the production of heterologous proteins with a potential as vaccines, therapeutics or enzymes. The expression system used includes a strong regulated promoter and has allowed a high-level production of recombinant *Leuconostoc mesenteroides* β-galactosidase, LacLM (Madsen et al., 1999).

With the development of microorganisms as cell factories for the production of heterologous proteins, a number of genetic tools for improved gene expression have been established. These include strong promoters, high copy number vectors, optimised codon us-

age and improved production strains, and their use has resulted in an increase of production lev ls.

Using these optimised tools, secretion of heterologous proteins into the culture supernatant might represent a limiting step. Therefore, the molecular knowledge of protein secretion is also emerging as a subject of applied research. To facilitate downstream processing of recombinantly produced protein, secretion of the protein is generally desired. To achieve efficient secretion of heterologous gene products it is required that constructs are used in which the gene coding for the desired gene product is operably linked to a gene coding for an effective signal peptide that can be recognised by the signal peptidases of the host cell.

The process of secretion in bacteria includes events that occur just after translation of the mRNA, i.e. the subsequent recognition of the signal peptide (SP) in the nascent unfolded polypeptide chain by the Sec apparatus and cleavage by signal peptidase upon translocation through the cell membrane.

The Sec-dependent pathway is the best studied system for protein export. Although it is known that virtually all proteins exported via this mechanism require a SP, it is not clearly understood how the structure of the SP interacts with the different components of the secretion machinery in the cell. The recent characterization of a Sec-independent pathway that is conserved between *E. coli* and plants illustrates the fact that proteins are exported through a number of distinct pathways (Settles and Martienssen, 1998; Stephens 1998). The mechanisms involved normally require the presence of sequence motifs in the exported protein.

SPs are the N-terminal extensions present in Sec-dependent secreted proteins. The structure of a typical SP includes three distinct regions: (i) an N-terminal region that contains a number of positively charged amino acids, lysine and arginine; (ii) a central hydrophobic core and; (iii) a hydrophilic C-terminus that contains the sequence motif recognised by the signal peptidase (von Heijne 1990). Despite structural similarities, large sequence variation is observed between different SPs. This variation has recently been related to specific targeting of the secreted proteins (Martoglio and Dobberstein, 1998).

Studies of secretion in *Escherichia coli* have shown the influence of the hydrophobic core region of SP on efficient processing. SPs with highly hydrophobic core regions supported

a high rate of transport even when an altered N-terminal region with negative charge is used (Izard t al., 1996). PhoA has been used as a model protein for detailed secretion studies in this bacterium. The effect of the removal of helix-breaking residues (Gly or Pro) can be compensated by increased hydrophobicity (Izard et al., 1995). In competition experiments, two identical SPs were placed N-terminal to PhoA and the rate of utilization of either SP was shown to be dependent on small increases in the hydrophobicity of one of the SP (Chen et al., 1996). Moreover, it was shown that a reduced negative charge at the amino terminus resulted in a lower overall affinity for the transport pathway (Izard et al., 1996).

The characterisation of numerous extracellular proteins has allowed development of a method for the prediction of the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms including Gram-positive and Gram-negative prokaryotes, and eukaryotes (Nielsen et al., 1997). The method involves a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks. The use of this method permits the preliminary design and analysis of SP derivatives prior to their construction and test *in vivo*.

Proteins that are targeted for secretion include a signal sequence or signal peptide (SP) at the N-terminus. SPs are recognised and cleaved by a leader or signal peptidase, a component of the secretion machinery of the cell, during translocation across the cell membrane (Martoglio and Dobberstein, 1998). SPs are normally 25 to over 35 amino acids (aa) in size in Gram-positive bacteria. SPs do not share sequence homology, but are often composed of an amino terminus that includes one or more basic aa, a central hydrophobic core of seven or more aa, and a hydrophilic carboxy terminus containing the motif that is recognized by signal peptidases (Martoglio and Dobberstein, 1998). A survey of available SPs from *L. lactis* suggested the use of the SP from Usp45, the major secreted lactococcal protein (van Asseldonk et al., 1990). This SP was reported to be functional in the secretion of several heterologous proteins in *L. lactis* (van Asseldonk et al., 1993).

Traditional strategies for the identification of SPs in *L. lactis* have followed the construction of genomic libraries in a vector carrying a promoterless reporter gene. In general, work in Gram-positive bacteria has involv d the use of reporter genes with a demonstrated functionality for the identification of SPs in Gram-negative bacteria. These reporters include BlaM, the *E. coli* β-lactamas (Sibakov et al., 1991; Perez-Martinez et al., 1992). The use of BlaM for the identification of *L. lactis* SPs implied a limitation on the

possibility of direct screening in *L. lactis* and this has been assumed to be due to differences in codon usage and protein folding requirements for BlaM (Pouquet et al., 1998). Therefore, primary screening of Gram-positive bacterial genomic libraries has up till now been carried out in *E. coli* and positive clones subsequently tested in *L. lactis* (Sibakov et al., 1991; Perez-Martinez et al., 1992) thereby imposing a tedious and labour consuming selection step for functionality in the primary host. A more appropriate secretion reporter, the extracellular β-amylase from *Bacillus licheniformis* has also been used in screening for lactococcal SPs, but following the same screening strategy (Perez-Martinez et al., 1992). These strategies resulted in the isolation of SPs of type I exclusively. Moreover, some of the functionality of the sequences identified was due to the presence of amino acid residues derived from the multiple cloning site in the vector. These amino acids matched the requirements for the C-terminal region of this type of SPs (Perez-Martinez et al., 1992).

However, it is desirable to dispose of a broad range of SPs in order to select, for specific purposes, such SPs that are suitable in a particular host cell or for the secretion of a particular gene product. A major objective of the present invention is therefore to provid a convenient method for direct isolation of lactic acid bacterial nucleotide sequences coding for signal peptides that are functional in a broad range of host cells including lactic acid bacterial cells, which method does not require an intermediate screening step in another species. A further objective of the invention is to provide a transposable element useful in the present method that permits to identify and locate, in a bacterial chromosome, sequences coding for SP. By using the novel method several novel lactococcal SPs w re identified, isolated and improved by mutagenesis.

SUMMARY OF THE INVENTION

Accordingly, the invention pertains in a first aspect to a method of constructing a transposon derivative for identifying in a lactic acid bacterium a DNA sequence coding for a signal peptide (SP), the method comprising the steps of (i) selecting a DNA molecule comprising a transposon including between its left terminus (LR) and its right terminus (RR) a sequence comprising a promoterless promoter reporter gene and a ribosome binding site (RBS), (ii) deleting from said DNA molecule a region located between the LR and the promoterless reporter gene to obtain a modified DNA molecule that has retained its transposability and its RBS, (iii) inserting into the remaining region located between the LR and the promoterless reporter gene of the resulting modified DNA molecule a unique restric-

tion site, and (iv) inserting into said unique restriction site a DNA sequence coding for a secretion reporter molecule, said DNA sequence coding for a reporter molecule is without a sequence coding for a SP, the thus obtained transposon derivative being without stop codons in-frame with the secretion reporter molecule thus permitting upon transposition translational fusions from upstream the LR.

In a further aspect there is provided a transposon derivative for the identification in a lactic acid bacterium of a DNA sequence coding for a signal peptide (SP), the molecule comprising the following elements: (i) a DNA molecule comprising a transposon element including between its left terminus (LR) and its right terminus (RR) a sequence comprising a promoterless promoter reporter gene and a ribosome binding site, the DNA molecule being without stop codons in the region upstream of the promoter reporter gene, (ii) a DNA sequence coding for a secretion reporter molecule, said DNA sequence is without a sequence coding for an SP.

In a still further aspect, the invention relates to a method of identifying in a lactic acid bacterium a DNA sequence coding for a signal peptide (SP), the method comprising the steps of (i) transforming a lactic acid bacterium with a transposon derivative as defined above and (ii) selecting from the transformed lactic acid bacterium, cells in which the promoterless promoter reporter gene is expressed and the gene product of the DNA sequence coding for a secretion reporter molecule is secreted.

In yet other aspects there are provided an isolated DNA molecule comprising at least part of a transposon derivative as defined herein and a DNA sequence coding for a signal peptide (SP) that is functional in a lactic acid bacterium and an isolated DNA sequence coding for a signal peptide that is derived from a molecule selected from the group consisting of SP10, SP13, SP307, SP310 and SP330 as described hereinbelow, and a derivative of any of said signal peptides having retained signal peptide functionality. It was found that such derivatives can have an enhanced secretion efficiency as compared to the corresponding wild type SPs.

Further aspects of the invention include: a recombinant plasmid comprising an isolated DNA molecule comprising at least part of a transposon derivative or an isolated DNA sequence according to the invention; a recombinant bacterium comprising a DNA sequence according to the invention; and use of such a bacterium for the production of a desired gene product.

DETAILED DISCLOSURE OF THE INVENTION

A major objective of the present invention is to provide a novel transposable element that permits the direct identification, i.e. without the use of an intermediate bacterial species, in the genome of a lactic acid bacterium of a sequence coding for a SP. Such an element is provided by the above method of constructing a transposon derivative for identifying in a lactic acid bacterium a DNA sequence coding for a signal peptide (SP).

In a first step of the method a DNA molecule is selected that comprises a transposon including between its left terminus (LR) and its right terminus (RR) a sequence comprising a promoterless promoter reporter gene and a ribosome binding site (RBS). In the present context, one such useful DNA molecule is one comprising the *Tn917* transposon or a derivative hereof. A particularly useful *Tn917* derivative is the plasmid pLTV1 that in addition to the *Tn917* transposon comprises a promoterless *lacZ* gene and a ribosome-binding site (RBS).

From the selected basic transposable DNA molecule a region, located between the LR and the promoterless reporter gene, is deleted to obtain a modified DNA molecule that has retained its transposability and its RBS, followed by inserting a unique restriction site into the remaining region located between the LR and the promoterless reporter gene and inserting into said unique restriction site a DNA sequence coding for a secretion reporter molecule that does not include a sequence coding for a SP.

By deleting the region located between the LR and the promoterless reporter gene it is achieved that the thus obtained transposon derivative is without stop codons in-frame with the secretion reporter molecule permitting, upon transposition, translational fusions from upstream the LR.

Among secretion reporters that can be used in *L. lactis* and other lactic acid bacterial species, genes coding for nucleases are presently preferred, including the *Staphylococcus aureus* nuclease (Nuc), a naturally extracellular protein that has been shown to be useful in *L. lactis* as secretion reporter (Poquet et al., 1998). Nuc is suitable for the screening for SPs since the protein is inactive intracellularly and its structur is remarkably simple (it is a monomer, lacks disulfide bonds). Furthermore, the codon usage in the *nuc* gene is ap-

propriate for high level expression in lactococci, and the plate assay for detection of secretion is not toxic, eliminating the need for replica plating.

In another aspect, the invention provides a novel transposon derivative molecule, which is useful in the above method for the identification in a lactic acid bacterium of a DNA sequence coding for a signal peptide (SP). Such a molecule comprises a first element in the form of a DNA molecule comprising a transposon element including between its left terminus (LR) and its right terminus (RR) a sequence comprising a promoterless promoter reporter gene and a ribosome-binding site. One example of a useful promoter reporter gen is the *lacZ* gene.

It is a significant feature of this first element that the DNA molecule is without stop codons in the region upstream of the promoter reporter gene that are in-frame with the secretion reporter molecule of the below second element, which permits, upon transposition of the transposon derivative, the expression of translational fusions from upstream the LR.

The transposon derivative molecule comprises, as a second element, a DNA sequence that codes for a secretion reporter molecule, said DNA sequence is without a sequence coding for a SP. In a presently preferred embodiment, the secretion reporter gene is a gene coding for a nuclease such as the *nuc* gene derived from *Staphylococcus aureus*.

In useful embodiments, the transposon derivative is derived from *Tn917* or a derivative hereof including pLTV1. A particularly useful transposon derivative according to the invention is pTn*Nuc*, the construction and function of which are described in details in the following examples. In addition to the above first and second elements, the transposon derivative may further comprise a selection marker, e.g. an antibiotic resistance gene or a mutation conferring auxotrophy against an essential nutrient component.

A major objective of the invention is to provide a method of identifying in a lactic acid bacterium a DNA sequence coding for a signal peptide (SP). The method comprises the steps of transforming a lactic acid bacterium with a transposon derivative molecule as described above and selecting from the transformed lactic acid bacterium, cells in which the promoterless promoter reporter gene is expressed and the gene product of the DNA sequence coding for a secretion reporter molecule is secreted.

It will be appreciated that an expression of the promoter reporter gene is an indication that the transposable element has been integrated into a gene of the lactic acid bacterial c ll at a position where it is operably linked to a promoter in the chromosome of the cell. Simultaneous screening for expression of the promoterless reporter gene and the secretion reporter gene on media which are indicative for the fusion product of the promoter reporter gene and the secretion reporter gene permits the direct identification of clones comprising a sequence coding for a functional SP.

In accordance with the invention, the lactic acid bacterium, which is used in the above as a source for functional SPs, can be of any species belonging to the group of bacteria generally referred to as lactic acid bacteria. This group includes *Lactococcus* spp. such as *Lactococcus lactis*, *Lactobacillus* spp. including as examples *Lactobacillus acidophilus* and *Lactobacillus plantarum*, *Leuconostoc spp.* such as *Leuconostoc mesenteroides*, *Oenococcus* spp. and *Streptococcus* spp.

In preferred embodiments, the transposon derivative is transposed randomly or quasi-randomly.

It will be appreciated that when clones comprising a sequence coding for a functional SP have been identified using the above method, such a sequence can be isolated in accordance with conventional techniques for isolating nucleotide sequences. Having isolated such a sequence it can, if desired, be inserted into homologous or heterologous species with the aim of improving or optimising the secretion of desired gene products.

Accordingly, the invention pertains in a further aspect to an isolated DNA molecule comprising at least part of a transposon derivative as defined above and a DNA sequence coding for a signal peptide (SP) that is functional in a lactic acid bacterium. In useful embodiments, such a DNA molecule comprises a sequence coding for a signal peptide comprising a signal peptidase I-recognition sequence or a signal peptidase II-recognition sequence. In this context, suitable DNA molecules include such molecules where the DNA sequence coding for a signal peptide is derived from a clone selected from the group consisting of SP10, SP307, SP310 and SP330 as described in the following examples, and a mutant th reof.

It has been found that it is possible to enhance the secretion efficiency of a desired gene product by operably linking the gene coding for the gene product to a mutant of a se-

quence coding for a naturally occurring SP. Such a mutation can be produced by conventional mutagenesis techniques such as mutagenesis by means of UV irradiation or by using chemical mutagens. However, site-directed mutagenesis has been found to be a convenient and effective means of producing SP mutants having improved functional characteristics. Such a mutagenesis may result in substitution, deletion or addition of one or more amino acid residue. In the following examples, a range of such mutated SPs are described, of which several show a higher secretion efficiency than the corresponding wild type SP. These mutants include those designated herein as 310mut1, 310mut2, 310mut3, 310mut4, 310mut5, 310mut6, 310mut7, 310mut8, 310mut10, 310mut11, 310mutA, 310mutB, 310mutC, 310mutA1, 310mutB1, 310mutD2, 310mutD7, 310mutE2, 310mutE11 and 310mutF2, respectively.

The invention also provides novel isolated DNA sequences coding for a signal peptide that are derived from a molecule selected from the group consisting of SP10, SP13, SP307, SP310 and SP330 as described herein, and a derivative of any of said signal peptides having retained signal peptide functionality, including DNA sequences derived from any of the above mutants. There are also provided recombinant plasmids comprising an isolated DNA molecule comprising at least part of a transposon derivative according to the invention or an isolated DNA sequence as described above. Such plasmids include a recombinant plasmid that is selected from the group consisting of Δ10::Nuc, Δ13::Nuc, Δ307::Nuc and Δ310::Nuc as described herein. In useful embodiments, the recombinant plasmid according to the invention comprises a regulatable promoter operably linked to the secretion reporter gene. The regulation of the promoter activity is preferably caused by growth condition factors for the host cell carrying the plasmid such as the growth temperature, the pH, the growth phase and changes of the nutrient composition of the medium occurring during growth of the host cell. In the present context, one useful promoter is the P170 promoter as described hereinbelow.

In a still further aspect, the invention pertains to a recombinant bacterium comprising a DNA sequence coding for a signal peptide that is derived from a molecule selected from SP10, SP13, SP310 and SP330, and a derivative of any of these SPs that has retained SP activity. In such a bacterium, this DNA sequence is preferably operably linked to a gene expressing a desired gene product whereby the gene product is secreted. Such a recombinant bacterium is any Gram-positive or Gram-negative bacterium that is used as host cell in the production of desired gene products. Typical examples of Gram-positive

bacteria include lactic acid bacterial species, *Bacillus* spp. and *Streptomyces* species and examples of Gram-negative bacteria include as a typical example *E. coli.*

The invention will now be illustrated in the following non-limiting examples and the drawings wherein

Fig. 1A illustrates the construction of pTn*Nuc*, a secretion reporter tool in *L. lactis.* Details of the construction of Tn*Nuc* are described in Example 1. Plasmids are not drawn to scale and only relevant features are shown. Restriction sites. A: *Aat*II; B: *Bsm*I; E: *Eco*RV; N: *Nsi*I; R: *Rsr*II; S: *Sma*I. The pPRA plasmids contain the 3.1 *Eco*RV fragment from pLTV1 spanning from the coding region of the *tet* gene (open tet arrow) to within the *lacZ* gene (stripped lacZ arrow) and including the left repeat of Tn*917* (filled LR box). This fragment (open LTV1 box) and the position of the LR (filled LR box) are shown in pPRA plasmids for clarity. The region deleted between the LR and *lacZ* is depicted as an open triangle in pPRA4B.

Fig. 1B illustrate details of the nt positions used in the deletion analysis of the Tn*917* derivative in pLTV1 are indicated together with the functionality of the different pPRA5 derivatives in transposition;

Fig. 2. summarises an analysis of secretion efficiency for selected *L. lactis* SPs. Concentrated (20-fold) supernatants of strain PRA157 (lane 1), PRA158 (lane 2) and PRA159 (lane 3) were run on a 16% Tricine gel and Coomasie stained. The volume loaded represents the total protein content from 100 µl (PRA157 and PRA158) or 50 µl (PRA159) of culture supernatant. The migration of molecular weight markers in kDa is shown to the left. The position of NucA and the corresponding full length protein (Δ13::Nuc, Δ307::Nuc and Δ310::Nuc is shown to the right). A weaker band with similar migration to Δ13::Nuc and present in all strains corresponds to Usp45, the major secreted protein from *L. lactis* (van Asseldonk et al., 1990);

Fig. 3. is an overview of the site-directed mutagenesis strategy for SP310 and Nuclease secretion. The profile obtained from the analysis of the wild type SP310 (SP310) using SignalP is shown abov . C, S and Y scores are the three parameters us d in SignalP for the identification of a SP. The arrow indicates the suggested main processing site between residues Ala34-Ala35, sequence alterations are underlined; and

Fig. 4 shows secreted nuclease yield in *L. lactis* strains containing an altered SP310. Supernatants from overnight cultures were concentrated about 50-fold by TCA-precipitation. A volume corresponding to 0.5 ml culture supernatant was run on a 14% SDS PAGE (Novex). Lane 1: strain PRA76 (Usp45SP-Nuc); lane 2: strain PRA159 (original construction with 60 aa from the SP310 sequence fused to the mature Nuc); lane 3: strain PRA162 (only the 35 aa of SP310 fused to Nuc); lane 4: strain PRA164 (310mut2-Nuc); lane 5: strain PRA170 (310mutB-Nuc); lane 6: strain PRA250 (310mut6-Nuc); The migration of molecular weight markers is indicated to the left in kDa.

EXAMPLE 1

The development of Tn*Nuc* and its use for the isolation of novel secretion signals in *Lactococcus lactis*

Abbreviations: aa: amino acid(s); *B.*: *Bacillus*; bp: base pair; *E.*: *Escherichia coli*; Em: Erythromycin; *L.*: *Lactococcus*; LR: left repeat; *nuc*: Nuclease coding gene; Nuc: Nuclease protein; nt: nucleotide; *S.*: *Staphylococcus*; SP(s): signal peptide(s) or secretion signal; *St.*: *Streptococcus*

1.1. Abstract

The construction of a new *Tn917*-transposon derivative, termed Tn*Nuc*, which includes the *Staphylococcus aureus* nuclease gene (*nuc*) as a reporter for secretion is described. Transposition of Tn*Nuc* into the *L. lactis* chromosome permits the generation of fusions in-frame with the *nuc* gene. Tn*Nuc* also includes *lacZ*, a reporter used for identification of relevant clones from the library, i.e. clones with Lac$^+$ phenotype resulting from transposition of Tn*Nuc* into a functional gene on the *L. lactis* chromosome. The presence of a functional signal sequence at the upstream flanking region of the left repeat of the transposed element results in the detection of nuclease activity using a sensitive plate assay. Tn*Nuc* was used for the identification of novel secretion signals from *L. lactis*. The sequences identified included known and unknown lactococcal secreted proteins containing either a signal peptidase-I recognition sequence or a peptidase-II recognition sequence. In one case, the gene identified codes for a transmembrane protein. The identified sequences were used to study functionality when located in a plasmid under the control of the pH and

growth phase-dependent promoter P170 (Madsen et al. 1999). In all cases concurrent s - cretion of nuclease was observed during induction of P170 in a chemostat.

1.2. Materials and methods

(i) Strains and growth conditions

Escherichia coli K-12 strain DH10B (Grant et al., 1990) grown in LB supplemented, if appropriate, with 100 μg/ml ampicillin or 200 μg/ml erythromycin (Em), at 37°C was used for cloning purposes, rescue of plasmid DNA and propagation of plasmid DNA. *Lactococcus lactis cremoris* strain MG1614 (Gasson 1983) was used in experiments involving construction of transposon insertions, screening, analysis of transposon insertions and plasmid rescue of DNA adjacent to site of insertion. *L. lactis* subsp. *cremoris* strain MG1614 (Gasson 1983) were used for analysis of isolated translocation signals. *L. lactis* strains were grown in GM17 or ArgM17 (Israelsen et al., 1995) at 30°C supplemented, if appropriate, with 1 μg/ml erythromycin (GM17Em or ArgM17Em). In fermentor experiments, a defined medium, 3 x SAIV (Jensen and Hammer, 1993) was used and pH was maintained using KOH.

Transformation of bacteria was performed by electroporation, according to published procedures for *E. coli* (Sambrook et al., 1989) and *L. lactis* (Holo and Nes 1989), respectively.

(ii) Plasmid and Tn*Nuc construction*

The strategy for the construction and analysis of deletion derivatives of Tn*917*-LTV1 (Camilli et al., 1990) is illustrated in Fig. 1. The 3.1 kb *Eco*RV fragment from pLTV1, spanning from the 3' end of the *lacZ* gene to the coding region of the *tet* gene (positions 12208 to 15335) was subcloned into *Eco*RV-digested pBluescript SK- (Stratagene), resulting in pPRA2. Plasmid DNA from pPRA2 was used as a template for PCR using primer PSS1b (5'-CGATGAATGC *CGGACCG*AAT TGATACACTA ATGCTTTTAT ATAGGG-3'(SEQ ID NO:1) containing restriction site for *Bsm*I (underlined) and *Rsr*II (italics), respectively; position 13374-13348 in pLTV1) and primer PSS14 (5'-GTGTAGTCGG TTTATGCAGC-3'(SEQ ID NO:2); position 12672-12691 in the *lacZ* gene). The amplified 720-bp fragment was digested with *Bsm*I and *Aat*II (unique site in the 3,1 kb pLTV1 fragment in pPRA2, Fig. 1) and cloned into *Bsm*I and *Aat*II-digested pPRA2, resulting in pPRA3. Four different PCR products (designated 2A to 2D) were amplified using pPRA2 DNA as template. PCR was carried out using primer PSS3 (5'-CACACATACC AATACATGC-3' (SEQ ID NO:3); position 14391-14373 in pLTV1, see Fig. 1) in combination with the following primers containing a unique *Rsr*II site (italics): (i) for 2A, primer PSS4 (5'-GCAT*CGGTCC GT*AGGCGCTC GGGACCCC-3' (SEQ ID NO:4), position 13665 to 13647 in pLTV1); (ii) for 2B, primer PSS6 (5'-GCAT*CGGTCC* GTTCTTATCG ATACAAATTC CTCG-3' (SEQ ID NO:5), position 13648 to 13626 in pLTV1); (iii) for 2C, primer PSS8 (5'-GCAT*CGGTCC GA*AATTTTTA AATCTATTTC TTATC-3' (SEQ ID NO:6), position 13633 to 13608 in pLTV1) and (iv) for 2D, primer PSS10 (5'-GCAT*CGGTCC GT*AAATGTAC AAAATAACAG CGAAAT-3' (SEQ ID NO:7), position 13613 to 13589 in pLTV1).

Fragments 2A to 2D were digested with *Rsr*II and *Nsi*I (a unique *Nsi*I site in the 3.1 kb *Eco*RV fragment from pLTV1, see Fig. 1) and cloned into likewise digested pPRA3, resulting in pPRA4A to pPRA4D, respectively. pPRA4A to pPRA4D were digested with *Eco*RV and the 2.8-2.9 kb inserts were cloned into *Eco*RV-digested pLTV1 to replace the original 3.1 kb fragment, resulting in plasmids pPRA5A tp pPRA5D (Fig. 1).

pPRA5B was chosen to construct Tn*Nuc*. Using pBS::Nuc (Le Loir et al., 1997) as DNA template and primers PSSnuc1 (5'-GCAT*CGGACC GT*CACAAACA GATAACGGCG-3' (SEQ ID NO:8), *Rswr*II site in italics) and PSSnuc2 (GCAT*CGGTCC GC*ATTATTGA CCTGAATCAG-3' (SEQ ID NO:9), *Rsr*II site in italics), a 531 bp fragment was obtained. Digestion with *Rsr*II and subsequent ligation into likewise treated pPRA5B was carried out

and resulted in pTn*Nuc*. The *nuc* fragment codes for the NucB form of the protein (Poquet et al., 1998).

Transposon insertions were made by transforming *L. lactis* with the vector carrying the Tn*917* derivative and growing these with erythromycin selection. Plates with transformants were either replica plated on X-gal plates as decribed by Israelsen et al. (1995) followed by Nuclease plate (Nuc) assays of $LacZ^+$-klones or a library of transposon insertions was generated by plating transformed *L. lactis* with high density on 140 mm petri dishes each with 200 ml SGM17 agar suplemented with 1 µg/ml Em. Plates were incubated 4 days at 30°C and clones were pooled by resuspending colonies in GM17Em and 17% glycerol. The constructed library was frozen in small aliquots. An aliquot was subsequently plated at a density of c. 500 cfu/plate on either GM17Em or ArgM17Em. Nuc assays were then performed on colonies to identify Nuc secreting clones.

For the analysis of the functionality of the isolated SPs on plasmid, pAMJ206 was used. pAMJ206 contains the *L. lactis* regulated promoter P170 (Madsen et al., 1999) located upstream of the ribosome binding site (RBS) from pAK80 (Israelsen et al., 1995). Uniqu *Bgl*II and *Sal*I sites are conveniently located just downstream of the RBS. A PCR fragment was obtained from pBS:Nuc (Le Loir et al., 1997) using primers Nuc1 and Nuc2 (respectively, 5'-GG*AAGATCT*T CACAAACAGA TAACGGC-3' (SEQ ID NO:10) and 5'-ACG-C*GTCGAC* GAATTCGATC TAAAATTAT AAAAGTGCC-3' (SEQ ID NO:11) restriction sites in italics), digested with *Bgl*II and *Sal*I and ligated into pAMJ206, resulting in pΔSPNuc. This plasmid was used for the construction of plasmid pPRA157, pPRA158 and pPRA159 as follows. In pPRA157, a PCR fragment was amplified from chromosomal DNA of *L. lactis* MG1614 using primer PSScluA-A and PSScluA-B (respectively, 5'-GCAT*CCCGGG* TCTAGATTAG GGTAACTTTG AAAGGATATT CCTCatgAAA AAAACATTGA GAGACCAGTTACTTG-3' (SEQ ID NO:12) and 5'-GCAT*AGATCT* ACTCCAACTA TCACCTGTTG CATTTGCTC-3' (SEQ ID NO:13), restriction sites, *Sma*I and *Bgl*II in italics, the sequence from the expression vector pAMJ203 is underlined and the *cluA* gene ATG start codon is shown in lower case). This fragment spans from the start codon of the *cluA* gene to a position 1200 bp downstream. At this position, insertion of Tn*Nuc* was observed in clone SP13. This fragment was cloned into *Sma*I-*Bgl*II digested pΔSPNuc resulting in an in-frame fusion protein CluA::Nuc containing just two aa differences (Arg-Ser, derived from the cloning sites) to the protein produced in SP13.

Construction of pPRA158 and pPRA159 was carried out similarly, but cloning a PCR fragment corresponding to the first 120 bp of the coding region for the genes inactivated by Tn*Nuc* insertion in clone SP307 (fragment amplified using primers PSS307-A, 5'-GCAT*CCCGGG* TCTAGATTAG GGTAACTTTG AAAGGATATT CCTCATGAAT AAATCAAAAA TTATTGCTTT CTCTGC-3' (SEQ ID NO:14) and PSS307-B, 5'-GCATAGATCT ATCAATGGAA TTAACATCAG CTGCCATGC-3' (SEQ ID BO15), respectively, *Sma*I and *Bgl*II sites in italics) and SP310 (fragment amplified using primers PSS310-A, 5'-GCAT*CCCGGG* TCTAGATTAG GGTAACTTTG AAAGGATATT CCTCATGAAA TTTATAAAAA AAAGAGTTGC AATAGCC-3' (SEQ ID NO:16) and PSS310-B, 5'-GCAT*AGATCT* GTTATCATTA AAATCACTCC GATTAAGAG-3' (SEQ ID NO:17), respectively, *Sma*I and *Bgl*II sites in italics), respectively in pΔSPNuc.

Additionally, strain AMJ627 was constructed by cloning a PCR fragment containing the N-terminal 29 aa that include the SP from Usp45 (SP_{Usp}, amplified using primers Usp1 (5'-TAGTAGGATC *CCGGG*TCTAG ATTAGGGTAA CTTTGAAAGG ATATTCCTCatg AAAAAAAA GATTATCTCAGC-3' (SEQ ID NO:18), *Sma*I site in italics, *usp45* ATG start codon in lower case) and Usp2 (5'-ACGCGTCGAC CTGCAG*AGAT CT*TGTGTCAG CGTAAACACC-3' (SEQ ID NO:19), *Bgl*II site in italics) into *Sma*I-*Bgl*I-digested pΔSPNuc. All plasmid constructions were confirmed by sequencing the relevant regions.

(iii) Nuclease assay

Nuclease assay on plates was performed by the colony overlay method as described by Lachica et al. (1971), with the following modifications: 0,1% sonicated herring sperm DNA and 40 μM $CaCl_2$ was used in overlay and 1% agar was substituted with 0.6% agarose. Colonies were grown on GM17Em, ArgM17Em or LBEm plates with 0,3% glucose. The plates were incubated for 30 minutes to 6h at 37°C after solidification of overlay. Nuclease-secreting colonies developed as clear orange zone on a blue-green background.

(iv) Insertional mutagenesis with Tn917 and derivatives

Transposon insertion experiments were performed by transforming *L. lactis* with the vector carrying the Tn*917* derivative and growing these with erythromycin selection. Primary transformants were replica plated on X-gal plates as decribed by Isra Isen et al. (1995) followed by Nuclease plate assays of $LacZ^+$-clones. Alternatively, a library of transposon insertions was generated by plating transformed *L. lactis* with high density on 140 mm petri dishes each with 200 ml SGM17 supplemented with 1 μg/ml Em. Plates were incu-

bated 4 days at 30°C and clones were pooled by resuspending colonies in GM17Em and 17% glycerol. The constructed library was frozen in small aliquots. Aliquots were subsequently thawed and plated at a density of c. 500 cfu/plate on either GM17 or ArgM17 supplemented with Em. Plate assay was then performed on colonies to identify clones secreting nuclease.

(v) Pulsed field gel electrophoresis

PFGE of *Sma*I-digested chromosomal DNA from *L. lactis* clones with integrated transposons was carried out as described (Israelsen and Hansen, 1993) to test the random distribution of Tn*Nuc*.

(vi) Plasmid rescue

DNA from regions flanking the integrated Tn*nuc* transposon was characterised as follows. For the DNA region flanking the transposon LR, chromosomal DNA (2 μg) was digested with *Eco*RI, religated in a large volume (200 μl) to favour intramolecular ligation and transformed into *E. coli* DH10B. For the DNA region adjacent to the RR, chromosomal DNA was digested with either *Mlu*I or *Bsi*WI before religation and transformation of *E. coli* DH10B.

(vii) Protein characterization

Alternative culture supernatants were concentrated 20- to 30-fold using the Phenol-Ether procedure (Sauvé et al., 1995). Samples were run on 16% Tricine gels (Novex), according to the manufacturer. The gels were stained overnight using the colloidal Coomasie staining kit (Novex). The Mark 12 Wide Range Standard was used to estimate molecular sizes.

(viii) DNA sequencing and computer analysis

Plasmid constructions and rescued plasmid DNA were sequenced using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham), Cy5-labelled primers and an ALFexpress DNA Sequencer (Pharmacia Biotech). DNA sequence data were analysed using the Wisconsin package from the Genetics Computer Group, Inc. Possible secretion signals were analyzed using the SignalP WWW server (Nielsen et al. 1997).

(ix) Fermentation

Fermentation experiments were carried out using bench top fermentors (Applikon) containing 1 litre of medium and set to operate at 30°C and to maintain pH above 5.2.

1.3. Results

(i) Identification of the minimal region required for transposition of Tn917 in Lactococcus lactis.

Transposition of the Tn*917* derivative included in pLTV1 was demonstrated during the search for regulated promoters in *L. lactis* (Israelsen et al., 1995). This derivative contains a promoterless *lacZ* gene at the transposon left terminus and a ribosome binding site derived from the *Bacillus subtilis spoVG* gene. The *SpoVG::lacZ* sequence is inserted 278 bp from the end of Tn*917*, at a position where this insertion does not abolish transposition (Youngman, 1987). In order to develop a transposon-based screening tool for the identification of secretion signals from *L. lactis*, a reporter gene encoding a secreted protein must be inserted at the left terminus of the element, to allow the generation of in-fram fusions with genes encoding secreted proteins upon transposition. This requires a short distance from the left terminus of Tn*917* together with the avoidance of stop codons that would prevent in-frame fusions with the reporter gene. Plasmids pPRA5A to pPRA5D were constructed as derivatives of pLTV1, each containing a small deletion that spans from a position within (pPRA5A) or adjacent to (pPRA5B to pPRA5D) the LR of Tn*917* to the region just upstream of *spoVG::lacZ* (Fig. 1). A unique *Rsr*II site was introduced in all derivatives upon deletion. *L. lactis* MG1614 was transformed using pLTV1 and pPRA5A-D. Transformants were grown through four rounds of replica plating in GM17Em plates. A large number of the initial transformants ceased to grow during replica plating. These corresponded presumably to isolates containing a poorly free replicating plasmid that was lost during subsequent growth on plates, rendering them Em-sensitive. Stable Em-resistant clones represent transposition of the Tn*917* derivative into the *L. lactis* chromosome, although the possibility of integration of the plasmid carrying the transposon cannot be excluded in a fraction of the clones (Israelsen et al., 1995). Transformation of *L. lactis* MG1614 with pLTV1, pPRA5B, pPRA5C and pPRA5D r sulted in a similar frequency of stable transformants. However, no stable transformants were obtained using pPRA5A. The Tn*917* derivative in pPRA5A contains a partial deletion of the LR of the element. Al-

teration of this structural feature may be responsible for the observed lack of transposition in *L. lactis*. Sequence integrity in this r gion is required to delimit the boundaries of the element and high sequence homology is observed among Tn*3*-related elements like Tn*917* (Sherrat et al., 1989).

In *L. lactis* MG1614, transformation with pLTV1 resulted in about 15% blue colonies among the stable Em-resistant clones on plates containing X-gal (Israelsen et al., 1995). Blue colonies result from Tn*917* transposition downstream of a promoter in the *L. lactis* chromosome. No significant differences in the frequency of blue clones were observed with pPRA5B, pPRA5C or pPRA5D, compared to pLTV1 (data not shown) indicating that these derivatives were functional in *L. lactis*.

Since pPRA5B included the largest deletion and retained functionality, it was chosen to clone a secretion reporter, the *S. aureus nuc* gene.

(ii) Construction of Tn*Nuc, a tool for the identification of signal peptides in L. lactis.*

A PCR fragment including the entire *nuc* coding region downstream of the SP (see s c-tion 2.2) was cloned into the unique *Rsr*II site of pPRA5B, resulting in pTn*Nuc* (Fig. 1). In this construction, stop codons in-frame with the *nuc* gene are avoided allowing translational fusions from upstream the LR. The new transposon, named Tn*Nuc*, was used for the construction of a collection of mutants in *L. lactis* MG1614. Tn*Nuc* retains the original *lacZ* coding region and ribosome binding site. This additional feature of Tn*Nuc* provides a phenotypic trait (Lac^+) to reveal the presence of promoter activity from sequences upstream of the LR upon transposition, regardless of the gene function. Thus, primary screening for blue colonies identifies *L. lactis* clones where true transposition occurs and where transcription into Tn*Nuc* occurs. Among them, a proportion of clones is expected to contain Tn*Nuc* adjacent to the 5' end of a gene encoding a secreted protein.

(iii) *Construction and screening of a collection of* Tn*Nuc integrants*

Two independent transformation experiments of *L. lactis* MG1614 were carried out with pTn*Nuc*. Since identification of a functional signal peptide requires both an active promoter and a gene encoding a secreted protein, initial screening focused on transposition events leading to expression of *lacZ*, excluding integration events not resulting in a fusion with a promoter in the correct orientation. In the first experiment, 147 Lac^+ clones w r

isolated and assayed for nuclease secretion using the plate overlay assay. A number of clones that formed dark blue colonies on X-gal plates showed weak Nuc activity on plates. These clones represented transposition of Tn*Nuc* into a strongly expressed gene, and the low level of Nuc on plates might be the result of limited cell lysis and not actual secretion. Twelve clones showing a weak Lac$^+$ phenotype and a low level of Nuc simultaneously were selected for further analysis, since in these cases true secretion of Nuc was more conceivable.

In the second type of experiments, an improved procedure was devised to reduce the elaborate and sequential screening mentioned above and to allow for the recovery of clones that would be lost during replica plating of primary integrants due to limiting promoter activity resulting in white colonies on X-gal plates. In this procedure, primary transformants were plated on SGM15Em plates and incubated for 4 days. This long incubation served the purpose of selection for true and stable Em-resistant integrants. Colonies were pooled and stored as a library that contained approximately 10^4 independent transposants. Subsequent analysis of the library was carried out using the Nuc assay on plates. Nuc$^+$ clones were then tested for LacZ activity. Out of 108 Nuc$^+$ clones obtained, 20 clones showing either (i) a strong Nuc$^+$ phenotype or (ii) a weak Nuc$^+$ together with a weak Lac$^+$ phenotype were selected for further analysis. These latter clones were assumed to include a Tn*Nuc* integration into a functional *L. lactis* gene.

In order to study the distribution of Tn*Nuc* in *L. lactis* chromosome, PFGE was carried out on *Sma*I-digested chromosomal DNA from 49 independent LacZ$^+$ clones. The presence of Tn*Nuc* introduces two adjacent *Sma*I sites, included in the Tn*Nuc* sequence (Fig. 1), in the *L. lactis* chromosome. Thus, the disappearance of a single SmaI fragment from the PFGE profile of *L. lactis* MG1363 and the presence of two novel *Sma*I bands demonstrate the presence of Tn*Nuc*. Among the clones examined, 35 showed the presence of Tn*Nuc* in the largest, 600 kb *Sma*I chromosomal fragment. This frequency (71%) is consistent with the previously reported transposition frequency (60%) of a similar Tn*917* system, TV32, into the same chromosomal region in *L. lactis* MG1614 (Israelsen and Hansen, 1993). Two clones had one or two copies of Tn*Nuc*, respectively, in the 310 kb *Sma*I chromosomal fragment. Tn*Nuc* transposition into the 280 kb, 140 kb and 120 kb *Sma*I chromosomal fragments was observed for 3, 2 and 2 clones, respectively. For the remaining 6 clones, no apparent change in the PFGE profile was detected. Insertion of Tn*Nuc* in one of the smaller *Sma*I fragments is assumed since size change for these fragments would not be detected under the conditions used for PFGE (Israelsen and

Hansen, 1993). Thes results confirmed the quasi-random distribution of Tn*Nuc* transposition in *L. lactis*.

(iv) Sequence analysis of L. lactis genes coding for secreted proteins.

All 32 clones obtained showing a positive Nuc$^+$/Lac$^+$ phenotype were used in plasmid rescue experiments to characterize the genes affected by Tn*Nuc* transposition. In two cases, plasmid rescue in *E. coli* did not yield any transformants and the corresponding clon s were not analysed further. Chromosomal DNA (300 to 400 bp) flanking the LR of Tn*Nuc* was sequenced on the rescued plasmids. In two cases, the sequence obtained corresponded to pTn*Nuc*. Integration of pTn*Nuc* but not transposition may have occurred in these *L. lactis* clones. For the remaining 28 clones, a sequence from the *L. lactis* chromosome was identified adjacent to the LR of Tn*Nuc*, representing true transposition events. DNA analysis revealed the presence of stop codons in the sequence just upstream of the Tn*Nuc* insertion, in-frame with the *nuc* gene in 10 clones. Among the remaining 18 clones, SP13 and SP36 included a Tn*Nuc* insertion in the *cluA* gene. CluA is a transmembrane protein involved in cell aggregation between donor and recipient bacteria during lactococcal conjugation (Godon et al., 1994). CluA has indeed a typical signal peptide, targ t for the signal peptidase I (Table 1). Nine clones were identified which contained a Tn*Nuc* insertion at 5 different positions within the same gene. This gene encodes a putative protein containing a signal peptide. A representative clone from this group, SP310, showed strong Nuc activity and low LacZ activity, indicating an effective signal sequence responsible for the secreted Nuc.

Table 1. Analysis of expression and secretion of selected Tn*Nuc* integrants. Homology search and type of signal peptide.

Clone	No. isolated[1]	Nuc activity[2]	LacZ activity[3]	Protein or putative homologue	Signal Peptide Type[4]
SP10	1 (1)	+	+++	PA243[5], unknown	I
SP11	2 (2)	+++	++	Unknown	-
SP13	2 (2)	+	+	CluA (Godon et al. 1994)	I
SP240	1 (1)	++	+	DexB[6]	ND
SP307	1 (1)	+++	+++	Hyaluronan synthas [7]	II

SP310	9 (5)	++	++	Unknown	I
SP323	1 (1)	+	+	Membrane transporter[8]	ND
SP330	1 (1)	+	+	Unknown	-

[1]The number of independent clones with a Tn*Nuc* insertion at the same locus is shown. Also the number of different locations within the target gene are given in brackets.

[2]Nuc activity was scored on plates using the overlay assay (see section 2.3).

[3]LacZ activity was scored on plates containing X-gal.

[4]Signal peptide analysis using the SignalP neural network (Nielsen et al., 1997); I: signal peptide type I; II: signal peptide type II (lipoprotein).

[5]Identical gene interrupted than in the previously isolated transposon integrant PA243 (Israelsen et al., 1995)

[6]*Streptococcus mutans dexB*, encodes an exoglycosylase involved n the metabolism of extracellular starch (Whiting et al., 1993).

[7]Hyaluronan synthase (HAS) from *Streptococcus equilisimus* (Ashbaugh et al., 1998).

[8]Homology to cation transporter proteins from *E. coli.*

ND: Sequence not available for the 5' end of the relevant gene.

Clone SP10 contained Tn*Nuc* insertion in a chromosomal locus adjacent to the *thr* operon that was previously identified during the search for regulated lactococcal promoters using pLTV1 (Madsen et al., 1996). SP10 showed strong promoter activity (LacZ), as it was reported for the pLTV1 integrant in the same locus, PA234 (Madsen et al. 1996). The protein encoded contained a putative signal peptide (Table 1).

Clone SP307 harboured a Tn*Nuc* insertion in a gene encoding a homologue of the streptococcal hyaluronase synthase (HAS), involved in capsule synthesis (Ashbaugh et al., 1998). A lipoprotein signal peptide (von Hejne, 1989)) is present at the N-terminus of the encoded protein (Table 1).

Analysis of clone SP11 and SP25 revealed an insertion of Tn*Nuc* at two different positions, 103 and 49 bp, respectively from the ATG start codon of the disrupted gene. For SP25, the short sequence is not sufficiently long to conform to a SP. However, a favourable ratio between Nuc and LacZ activity was observed from both SP11 and SP25 (Table 1). The observed nuclease activity may be due to partial processing and secretion of the Nuc fusion protein. Alternatively, the inactivated gene may be responsible for a compo-

nent of the secretion machinery or the cell membrane, leading to increased secretion or protein leakage.

In two clones, SP240 and SP330, no SP was identified in the targeted gene. For SP240, a database search identified significant homology to the *St. mutans dexB* gene. *dexB* encodes an exoglycosylase involved in the degradation of extracellular starch (Whiting et al., 1993). Interestingly, no SP is found in DexB (Table 1). The mechanism underlying the observed Nuc secretion in SP240 and SP330 remains unclear.

Finally a gene encoding a membrane protein was the insertion target in clone SP323. The putative protein showed homology to Mg2+ transporters of *E. coli* (Table 1).

(v) Functional analysis of selected secretion signals on a multicopy plasmid in L. lactis.

Four SPs representing sequences from known (SP13) and previously unknown gen s (SP10, SP307 and SP310) and belonging to SPs recognized by signal peptidase I or II, were chosen for further analysis. To analyse secretion efficiency for the fusion partners identified with Tn*Nuc* in the wild type background, plasmid constructions were carried out to clone the minimal region (*i.e.*, the closest position of insertion to the corresponding ATG start codon, see section 2.2). Preliminary results showed that a CluA-Nuc protein fusion carrying an N-terminal CluA region including the first 60-150 aa did not yield detectabl amounts of secreted Nuc (data not shown). Therefore, a region including the N-terminal 400 aa of CluA corresponding to the closest position of Tn*Nuc* insertion to the 5' end of *cluA* identified herein was used for comparison (SP13).

For all other SPs, a 60 aa N-terminal region of the protein (SP10, SP307 and SP310) was introduced as a protein fusion with Nuc (hereafter called Δ10::Nuc, Δ13::Nuc, Δ307::Nuc and Δ310::Nuc, respectively), resulting in strain PRA156 (Δ10::Nuc) , PRA157 (Δ13::Nuc), PRA158 (Δ307::Nuc) and PRA159 (Δ310::Nuc). The Nuc fragment used in this study corresponds to the NucB form of the protein. This form is further processed into a 19-to-21 aa shorter form, NucA (Pouquet et al., 1998). Strains PRA156 to PRA159 include a pH and growth-phase dependent promoter, P170, for regulated expression (Madsen et al., 1999). P170-driven expression occurs during transition to stationary phase, when the growth medium is kept at pH~6.5. Fermentor experiments were carried out using the above strains, and samples were taken at the onset of the stationary phase where maximum production levels have been obtained (Madsen et al., 1999). Initial measur -

ments showed only background Nuc activity levels in culture supernatants of PRA156. This strain was therefore not analysed further.

Concentrated culture supernatants from PRA157, PRA158 and PRA159 were used to run SDS-PAGE. As shown, secretion of NucA was observed in all three strains (a 19-kDa band in lanes 1-3, Fig. 2). An additional band that corresponded in size to the full-length fusion protein was identified in PRA157 (a 42-kDa band) and PRA158 (a 22-kDa band; Fig. 2). In strain PRA159, two additional products of similar size (approximately 23 kDa) were detected, suggesting alternative processing sites of the SP in SP310). This possibility was supported by the identification of two putative processing sites of the primary aa sequence, at positions 27 and 34 from the initial Met, using SignalP (available at http://www.cbs.dtu.dk/services/SignalP, data not shown). Overall, the secretion efficiency was highest for PRA159 (Δ310::Nuc) and lowest for PRA157 (ΔCluA::Nuc). The samples were used for measurement of Nuc activity (Table 2).

As a comparison, strain AMJ627 was used. AMJ627 harbours a construction similar to the above, and that includes the full SP from Usp45 fused to Nuc (SP_{Usp}::Nuc; see section 2.2). The values obtained confirmed the efficiency of the SP from Usp45 in secretion. The SP used in PRA159 (Δ310::Nuc) secreted 67 mg/L Nuc, or about 60% compared to SP_{Usp}. Activity levels were lower for PRA158 (31%) and PRA157 (6%).

Table 2. Nuclease activity in culture supernatants of selected clones.

Strain (Fusion protein)[1]	Nuc activity	
	Total (mg/L)	mg/L x OD_{600}
PRA157 (ΔCluA::Nuc)	6.5	2.3
PRA158 (Δ307::Nuc)	34,1	10.0
PRA159 (Δ310::Nuc)	67,0	24.8
AMJ627 (SP_{Usp}::Nuc)	108,4	40.4

Stationary phas culture supernatants from fermentor xperiments were used.

[1]S secti n 2.2 for construction details

EXAMPLE 2

Molecular characterization and engineering of SP310, a signal peptide from *Lactococcus lactis*

2.1 Abstract

Among the signal peptides (SP) identified in Example 1, SP310 showed the highest level of secretion. However, the levels obtained were lower than those obtained using the signal peptide of Usp45 (SPUSP), the major secreted lactococcal protein. In this example is describes a site-directed mutagenesis approach for SP310 designed to improve secr tion levels and to study the requirements for Sec-dependent secretion in *L. lactis*. One of the mutants analyzed, SP310mut2, showed a secretion level similar to SPUSP, yielding more than 150 mg/L *Staphylococcus aureus* Nuclease (Nuc) in fermentor. This represents a 45% improvement with respect to the wild type SP310 sequence. The analysis of Nuc secretion in the mutants allowed the establishment of some of the requirements for efficient secretion in *L. lactis*. Common features for the *L. lactis* Sec-dependent secretion pathway differ from the features reported for *Escherichia coli*.

2.2. Materials and methods

(i) Strains and growth conditions

Escherichia coli K-12 strain DH10B grown in LB or TB supplemented, if appropriate, with 100 μg/ml ampicillin or 200 μg/ml erythromycin (Em) at 37°C was used for cloning purposes, rescue of plasmid DNA and propagation of plasmid DNA. *Lactococcus lactis cremoris* strain MG1363 (Gasson 1983) was used for analysis of SP. *L. lactis* strains were grown in GM17 (Israelsen et al., 1995) at 30°C supplemented, if appropriate, with 1 μg/ml erythromycin (GM17Em or ArgM17Em). In fermentor experiments, a defined medium, SAIV (Jensen and Hammer, 1993) was used and pH was maintained using HCl or NaOH. Transformation of bacteria was performed by electroporation, according to published procedures for *E. coli* (Sambrook et al., 1989) and *L. lactis* (Holo and Nes 1989), respectively.

(ii) Site-directed mutagenesis of SP310 and plasmid constructions

Primers PSS310-A (5'-GCAT*CCCGGG* TCTAGATTAG GGTAACTTTG AAAGGATATT CCTCATGAAA TTTAATAAAA AAAGAGTTGC AATAGCC-3' (SEQ ID NO:20), *Sma*I site in italics) and PSS310-B0 (5'-CTATTGGTTT GATTACGTCG GCTT*TCTAGA* TACG-3' (SEQ ID NO:21), *Bgl*II site in italics) were used to amplify the wild type SP310 sequence (herefter designated SP310) using pPRA159 DNA as template.The amplified fragment was digested with *Sma*I and *Bgl*II, purified from agarose gels and cloned.

For the construction of 310mut1, PSS310-A and PSS310-B1 (5'-GGTTCTATTG GTTCGATTAC GTCGGCTT*TC TAGA*TACG-3' (SEQ ID NO:22), *Bgl*II site in italics, mutation underlined) were used. PSS310-A and PSS310-B2 (5'-GTTATAGTAG TTAGGTTCTA CGAGTT---- --CGTCGGCTT*TCTAGA*TACG-3' (SEQ ID NO:23), *Bgl*II site in italics, mutations underlined and deletion shown as lines) were used to obtain 310mut2.

310mut3 was produced by using PSS310-A and PSS310-B3 (5'-CTATAAACAT TCAAAAAAAT GTTAT----- -TAGGGT--TTGTGACGAG TTCGTCGGCT T*TCTAGA*TAC G-3' (SEQ ID NO:24), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

310mut4 was constructed using PSS310-A and PSS310-B4 (5'-CTATAAACAT TCAAAAAAAT GTTAT----- -TAGGTT--- TTGGTTTGAT TACGTCGGCT T*TCTAGA*TAC G-3' (SEQ ID NO:25), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

310mut5 was obtained using PSS310-A and PSS310-B5 (5'-CTATAAACAT TCAAAAAAAT GTTAT----- -TAGGTT--- TTGGTTCGAT TACGTCGGCT T*TCTAGA*TAC G-3' (SEQ ID NO:26), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

310mut6 was constructed using primer PSS310-A and PSS310-B6 (5'-GTTATAGTAG TTAGGTTCTA CGAGTT---- --CGTCTATG A*TCTAGA*TAC G-3' (SEQ ID NO:27), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

310mut7 was obtained using primers PSS310-A and PSS310-B2ΔQ (5'-GTTATAGTAG TTAG---CTA CGAGTT---- --CGTCGGCT T*TCTAGA*TAC G-3' (SEQ ID NO:28), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

310mut8 was produced using primer PSS310-A and PSS310-B2ΔD (5'-GTTATAGTAG TTAGGTT--- CGAGTT---- --CGTCGGCT T*TCTAGA*TAC G-3' (SEQ ID NO:29), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

310mut10 was constructed using primer PSS310-A and PSS310-B21F (5'-CGTTATCGGT GCAAATAAAA AAACTATAAA CATTCAAAAA AATGTTATAG TAGTTAGGTT CTACGAGTT- -----CGTCG GCTT*TCTAGA* TACG-3' (SEQ ID NO:30), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

310mut11 was obtained using primer PSS310-A and PSS310-B22F (5'-CGTTATCGGT GCAAATAAAA AAACTATAAA CATAAAAAAA AATGTTATAG TAGTTAGGTT CTACGAGTT - -----CGTCG GCTT*TCTAGA* TACG-3' (SEQ ID NO:31), *Bgl*II site in italics, mutations underlined and deletion shown as lines).

For the construction of 310mutA, primer PSS310-AA (5'-CCT*CCCGGG*T CTAGATTAGG GTAACTTTGA AAGGATATTC CTCatgAAAT TTAATAAAAA AAGAGTTGCA ATAGCCTTGT TTATTGCTTT GATATTTGTA CTTTTTTTTC TTATATCATC-3' (SEQ ID NO:32), *Sma*I site in italics, ATG start codon in lower case and mutations underlined) and PSS310-B0 were used.

310mutB was obtained using primer PSS310-AB (5'-CCT*CCCGGG*T CTAGATTAGGG TAACTTTGAAA GGATATTCCTC atgAAATTTA ATAAAAAAAG AGTTCTTATA CTTTTGTTTA TTCTTTTGAT ATTTGTACTT TTTTTTCTTA TATCATC-3' (SEQ ID NO:33), *Sma*I site in italics, ATG start codon in lower case and mutations underlined) and PSS310-B0. 310mutC was obtained using primer PSS310-AC (5'-CCT*CCCGGG*T CTAGATTAGG GTAACTTTGA AAGGATATTC CTCatgAAAT TTAATAAAAA AAGACTTTTG CTTTTGCTTT TGCTTTTGCT TTTACTTCTT TTG-3' (SEQ ID NO:34), *Sma*I site in italics, ATG start codon in lower case and mutations underlined) and PSS310-BC (5'-GAAAATGAAG AAAACGAAAA CGAATATAGT AGTTAGGTTC TATTGGTTTG ATTACGTCGG CTT*TCTAGA*T ACG-3' (SEQ ID NO:35), *Bgl*II site in italics and mutations underlined).

310mutA1 was constructed using primer PSS310-AA and PSS310-B1 (5'-GGTTCTATTG GTTCGATTAC GTCGGCTT*TC TAGA*TACG-3' (SEQ ID NO:36), *Bgl*II site in italics and mutation underlined).

310mutB1 was obtained using primer PSS310-AB (5'- CCT*CCCGGG*T CTAGATTAGG GTAACTTTGA AAGGATATTC CTCatgAAAT TTAATAAAA AAAGAGTTCT TATACTTTTG TTTATTCTTT TGATATTTGT ACTTTTTTTT CTTATATCAT C-3' (SEQ ID NO:37), *Sma*I site in italics, ATG start codon in lower case and mutations underlined) and PSS310-B1.

Construction of 310mutD2 was performed using primer PSS310-AΔF (5'-GCAT*CCCGGG* TCTAGATTAG GGTAACTTTG AAAGGATATT CCTCatgAAA ---AATAAAA AAAGAGTTGC AATAGCC-3' (SEQ ID NO:38), *Sma*I site in italics, ATG start codon in lower case, mutations underlined and deletions shown as lines) and PSS310-B2.

310mutD7 was constructed using primer PSS310-AΔF and PSS310-B2ΔQ. 310mut E2 was obtained using primer PSS310-AΔN (5'-GCAT*CCCGGG* TCTAGATTAG GGTAACTTTG AAAGGATATT CCTCatgAAA TTT---AAAA AAAGAGTTGC AATAGCC-3' (SEQ ID NO:39), *Sma*I site in italics, ATG start codon in lower case and deletions shown as lines) and PSS310-B2.

Construction of 310mutE11 was performed using primer PSS310-AΔF and PSS310-B22F.

310mutF2 was constructed using primer PSS310-AK (5'-GCAT*CCCGGG* TCTAGATTAG GGTAACTTTG AAAGGATATT CCTCatgAAA TTTAAAAAAA AAAGAGTTGC AATAGCC-3' (SEQ ID NO:40), *Sma*I site in italics, ATG start codon in lower case and mutations underlined) and PSS310-B2.

In all cases, clones were introduced in *E. coli* by electroporation, and the presence of mutations in the 310 sequence was confirmed by sequencing both strands using a Thermo Sequenase fluorescently labelled primer cycle sequencing kit (Amersham), Cy5-labelled primers and an ALFexpress DNA Sequencer (Pharmacia Biotech). Plasmid DNA was subsequently introduced into *L. lactis* MG1363.

(iii) Protein analysis and SDS-PAGE

Culture supernatants were concentrated 20- to 30-fold using the Phenol-Ether procedure (Sauvé et al., 1995). Samples were run on 16% Tricine gels (Novex), according to the manufacturer. The gels were stained overnight using the Colloidal Coomassie Staining Kit

(Novex). The Mark 12 Wide Range Standard (Novex) was used to estimate molecular sizes.

(iv) Protein sequence analysis

Derivatives of SP310 were analysed using the SignalP WWW server available at http://www.cbs.dtu.dk/services/SignalP/index.html (Nielsen et al. 1997) to predict their suitability as SP.

(v) Fermentation

Fermentation experiments were carried out using bench top fermentors (Applikon), containing 1 liter of medium and set to operate at 30°C and to maintain the pH above 5.2.

2.3. Results

(i) An experimental setup for the analysis of secretion efficiency in L. lactis

In Example 1 a number of novel SPs were constructed using insertional mutagenesis with a Tn*917* derivative containing the *nuc* gene. Among these, SP310 was selected for further work since its use resulted in the highest yields of Nuclease (Nuc) secretion, when expression of the SP310-*nuc* gene fusion was driven by promoter P170 on a multicopy plasmid. Compared to the SP from Usp45 (SPUSP), the major secreted lactococcal protein, the Nuc secretion yield obtained using SP310 was significantly lower.

In order to improve the secretion level of SP310 and to allow for the easy identification of mutants with enhanced secretion of Nuc, the levels of Nuc in culture supernatants from overnight cultures grown in GM17 were compared to the level obtained during growth in fermentor in SAIV medium. As shown, the use of the minimal SP 35 aa wild type sequence including the Ala at position +1 (Ala^{+1}, the N-terminal aa of the processed protein) resulted in a secretion level of 5,67 mg/L of Nuc. This represents about 78% of the level observed using SPUSP (strain PRA76) after overnight growth in GM17 (Table 3). Total values of secreted Nuc were lower than the Nuc levels obtained in fermentor, but the relative efficiency of SP310 was in the same order of magnitude compared to SPUSP, i.e. over 58% (106 vs. 182 mg/L respectively). Thus, it was decided to measure Nuc secretion

in overnight culture supernatants grown in GM17 for the initial screening of SP310 mutants.

Overall, about 20-fold decrease in the total amount of Nuc secretion was obtained in GM17 overnight cultures compared to fermentors. This is mainly due to different regulation of the P170 promoter, the unequal physiological conditions and the media used. For SP310, 5,67 mg/L were measured in GM17 culture supernatants and 106 mg/L in fermentor. The results of the GM17 cultures are summarised in table 3.

Table 3. Amino acid sequences of SP310 and mutants, and secretion levels in GM17 overnight cultures.

Name	Sequence	Nuc (mg/L)	%
SP310	M K F N K K R V A I A T F I A L I F V S F F T I S S I Q D N Q T N A A E R S	5.67 ± 1.20	100
310mut1	M K F N K K R V A I A T F I A L I F V S F F T I S S I Q D N Q A N A A E R S	6.40 ± 0.66	113
310mut2	M K F N K K R V A I A T F I A L I F V S F F T I S S I Q D A Q - - A A E R S	7.03 ± 0.74	124
310mut3	M K F N K K R V A I A T F I A L I F V S F F T I - - I P - N T A Q A A E R S	6.16 ± 0.03	109
310mut4	M K F N K K R V A I A T F I A L I F V S F F T I - - I Q - N Q T N A A E R S	3.93 ± 0.72	69
310mut5	M K F N K K R V A I A T F I A L I F V S F F T I - - I Q - N Q A N A A E R S	3.90 ± 0.97	69
310mut6	M K F N K K R V A I A T F I A L I F V S F F T I S S I Q D A Q - - A D T R S	6.40 ± 0.49	113
310mut7	M K F N K K R V A I A T F I A L I F V S F F T I S S I - D A Q - - A A E R S	5.01 ± 0.08	88
310mut8	M K F N K K R V A I A T F I A L I F V S F F T I S S I Q - A Q - - A A E R S	3.44 ± 1.27	61
310mut10	M K F N K K R V A I A T F I F L I F V S F F T I S S I Q D A Q - - A A E R S	5.52 ± 1.33	97
310mutA	M K F N K K R V A I A L F I A L I F V L F F L I S S I Q D N Q T N A A E R S	2.64 ± 0.74	46
310mutB	M K F N K K R V L I L L F I L L I F V L F F L I S S I Q D N Q T N A A E R S	2.30 ± 0.50	41
310mutC	M K F N K K R L L L L L L L L L L L L L L I S S I Q D N Q T N A A E R S	1.99 ± 0.03	35
310mutA1	M K F N K K R V A I A L F I A L I F V L F F L I S S I Q D N Q A N A A E R S	3.60 ± 0.58	63
310mutB1	M K F N K K R V L I L L F I L L I F V L F F L I S S I Q D N Q A N A A E R S	2.21 ± 0.02	39
310mutD2	M K - N K K R V A I A T F I A L I F V S F F T I S S I Q D A Q - - A A E R S	6.31 ± 1.41	111
310mutD7	M K - N K K R V A I A T F I A L I F V S F F T I S S I - D A Q - - A A E R S	5.79 ± 1.03	102
310mutE2	M K F - K K R V A I A T F I A L I F V S F F T I S S I Q D A Q - - A A E R S	7.02 ± 0.17	124
310mutE11	M K - N K K R V A I A T F I F L I F V F F F T I S S I Q D A Q - - A A E R S	3.62 ± 1.15	64
310mutF2	M K F K K K R V A I A T F I A L I F V S F F T I S S I Q D A Q - - A A E R S	1.40 ± 0.25	25
SPUSP		7.27 ± 0.07	128

Nuclease yields from two independent growth experiments are shown as mg/L and as percentage of the activity shown by the wild type SP310 (100%). An open box in the SP310 sequence depicts the suggested hydrophobic core of the signal sequence. The values obtained for SPUSP (strain AMJ627) are also shown at the bottom. Th sequenc s 310mut0-310mutF2 are included as SEQ ID NO:41-62.

(ii) Site-directed mutagenesis of the L. lactis *SP310 signal sequence*

Initial analysis of the primary SP310 aa sequence was carried out using SignalP. Efficient bacterial SPs show an Ala at position -3, -1 and +1. The sequence of SP310 included a Thr, a relatively infrequent aa, at position -3 (Thr^{-3}). As shown, substitution of the Thr^{-3} residue to Ala^{-3} (in strain 310mut1) resulted in a significant increase in Nuc secretion (Table 3). It was evident that the C-terminal region of SP310 (Fig. 3), was unusually long and included a number of residues (position -10 to -2; Ser^{-9}, Ser^{-10}, Asn^{-5}, Gln^{-4}, Gln^{-7}, Asp^{-6} and Thr^{-3}) normally not present in this domain of Gram-positive SPs. Deletion of Thr^{-3} and Asn^{-2} and substitution of Asn^{-5} to Ala^{-3} was therefore incorporated into 310mut2. This mutant retains Ala at position -3, -1 and +1 (Fig. 3). Analysis of Nuc secretion in 310mut2 showed an increase of up to 24% compared to the wild type SP310 and also an increase with respect to the levels observed with 310mut1 in overnight cultures grown in GM17 (Table 3). These results confirmed that shortening of the C-terminal region and maintenance of Ala residues at the cleavage site resulted in a considerable improvement in Nuc secretion in *L. lactis*.

The analysis of SP from Gram-positive bacteria using SignalP predicted that a turn favoring aa (e.g. Gly or Pro) is often located at the position between the hydrophobic core and the C-terminal domain. In SP310, two Ser residues (Ser^{-10} and Ser^{-9}) are located in this region. We constructed a mutant lacking these two residues and Asp^{-6} (310mut4). These alterations resulted in a considerable reduction in the level of Nuc compared to SP310 (Table 3), suggesting that the presence of Ser and/or Asp in the C-terminal region of SP310 is a requirement for effective secretion in *L. lactis*. A single substitution in 310mut4 to incorporate an Ala residue at position -3 (in 310mut5) did not affect secretion efficiency (Table 3), strongly pointing out the essential role of these residues. However, substitution of Asn^{-2}, Gln^{-4} to more frequent aa found at these positions (Gln^{-2} and Thr^{-4}), together with a substitution of Gln^{-6} to Pro^{-6} in 310mut3 resulted in a higher level of secretion as compared to SP310, but lower than 310mut2. Pro is also found in this region in the SP of two major extracellular *L. lactis* proteins, PrtP and Usp45 (Table 4 below) and the results obtained with 310mut3 support the role of this aa in secretion. Interestingly, a double Ser is also present in this region in Exp2, another *L. lactis* extracellular protein whose SP was recently identified (Poquet et al., 1998).

Table 4. Signal peptide structure in *L. lactis*.

Protein	Positive Charge	Hydrophobic Core	Processing Region	Mature Protein
SP310	MKFNKKR	VAIATFIALIFVSFFTI	SSIQDNQTNA	AERS
310mut2	***MKFNKKR***	***VAIATFIALIFVSFFTI***	***SSIQDAQA***	***AERS***
Usp45	MKKKIIS	AILMSTVILSAAA	PLSGVYA	DTNSD
PrtP	MQRKKKG	LSILLAGTVALGALAVL	PVGEIQAKA	AISQQ
Exp1	MKNLIPKKIKQ	VGILVGALLMLLSVLPVNLL	GVMKVDA	DSSQTEV
Exp2	MKK	IAIIFCTLLMSLSVL	SSFAVSA	DTTTTTNN

Analysis of additional changes designed to shorten the C-terminal region by removing either Gln^{-7} (strain 310mut7) or Asp^{-6} (strain 310mut8) resulted in a significant decrease in secretion (Table 3). It remains unclear whether the length change or the alteration in charge in the C-terminal region is responsible for the decrease in secretion efficiency.

The N-terminal residues of the mature protein may also be important for effective processing by the secretion machinery. In the *L. lactis* Usp45 and Exp2, Asp and Thr are the first two aa in the processed protein (Table 4). Thus, a mutant was studied that includ d these two aa at position +1 and +2, preserving the sequence of 310mut2 in the SP. As shown, this mutant, 310mut6 secreted a slightly lower amount of Nuc compared to 310mut2, although the levels obtained were higher compared to SP310 (Table 3).

A series of mutants were constructed and characterized to study the role of the hydrophobic core in secretion in *L. lactis*. In *E. coli*, the hydrophobic core appears to effect an essential role in secretion, and increasing the hydrophobicity of this region compensates defects in either the N-terminus or the processing region (REF). Therefore, substitution of Ala^{-20} alone (in 310mut10) or in combination with Ser^{-15} (strain 310mut11) to Phe was analyzed. As shown, a correlation between the decrease in secretion efficiency and the number of Phe introduced was observed. Nuc secretion in strain 310mut11 was much lower than in 310mut2 and slightly lower than SP310 (Table 3).

In *E. coli* the presence of Leu in the hydrophobic region has also proven as an enhancer of secretion. A series of mutants were therefore constructed that included three, six or fourteen Leu residues in addition to the Leu^{-19} present in SP310. In 310mutA, 310mutB and 310mutC, Leu was introduced at different positions in the hydrophobic region maintaining the wild type sequence at the C-terminal region (Table 3). A large reduction in fficiency (46% compared to SP310) was observed in 310mutA, and even lower yields were obtained in 310mutB (41%) and 310mutC (35%), indicating that increasing amounts of Leu result in a gradual decrease in the secretion level. A modified version of 310mutA and 310mutB that incorporated also an Ala at position -3 (corresponding to the C-terminal region of 310mut1) was studied. In one of these mutants, 310mutA1, the level of secretion was somewhat higher (63% compared to SP310) indicating that conserved Ala positions in the processing region partially compensate the presence of moderate excess Leu in the hydrophobic core in *L. lactis*. However when six Leu were present (strain 310mutB1), the yield obtained was similar to 310mutB, indicating that the higher Leu content in the hydrophobic core cannot be compensated by the presence of Ala^{-3} (Table 3).

A series of mutants was constructed to investigate the influence of the N-terminal region in secretion. In this series, the C-terminal region of 310mut2 (or 310mut7) was used and removal of Phe or Asn from the N-terminal region was carried out, to increase the overall positive charge. In 310mutD2, removal of Phe^{-33} resulted in secretion levels higher than SP310 but lower than 310mut2 (Table 3). When the C-terminal region of 310mut7 was used an even lower yield was obtained, providing additional evidence that the lack of Gln^{-7} is attenuated in a SP carrying a shorter or more polar N-terminal region. Removal of Asn^{-32} (strain 310mutE2) resulted in maximal levels of secretion identical to 310mut2 (Tabl 3), suggesting a minimal role of this residue in efficiency. A dramatic decrease was observed when the hydrophobic and C-terminal region of 310mut11 were combined with the N-terminal region of 310mutD2 in strain 310mutE11, strongly confirming the main role of the hydrophobic core in the overall performance during secretion (Table 3). In strain 310mutF2, the N-terminal region was modified by substitution of Asn^{-32} to Lys, to increase the net positive charge. This alteration resulted in the lowest level of Nuc secretion observed among all mutants analysed (Table 3). Thus, the net charge of the N-terminal region of SP310 might represent the maximum allowed for efficient secretion in *L. lactis*.

(ii) Processing and secretion of Nuc using SP310 and selected mutants

Supernatants from overnight cultures of AMJ627, PRA159 and mutants PRA164, 310mutB and 310mutB were analysed in SDS-PAGE. In AMJ627, a strong band corresponding in size to the processed Nuc protein was observed, in addition to the main 45 kDa Usp45 protein (Fig. 4, lane 1). For PRA159, the gene fusion includes the full length SP310 and 25 additional codons corresponding to the N-terminus of the processed protein (Example 1). A main 19 kD band was observed that is the expected size of a full length Nuc with a 25 aa N-terminal extension. Minor bands in this preparation might correspond to further processing of the fusion protein into full-length Nuc (a 16 kDa band was detected; Fig. 4, lane 2). In strain PRA164, the presence of two major Nuc bands supported the alternative processing site suggested by the sequence analysis using SignalP (Fig. 3). A single Nuc band was detected in PRA164, 310mutB and 310mut6, indicating that a single processing site is used in these mutants. The relative amounts of Nuc were in agreement with the activity levels measured in these strains (Table 3).

(iii) Analysis of Nuc secretion in fermentor

As mentioned above, the production levels using the P170-dependent expression system of *L. lactis* are at least 20-fold higher during controlled growth in defined medium in fermentor as compared to the levels obtained in overnight cultures grown in rich GM17 medium. Therefore, the wild type SP310 and three mutants representing maximal (310mut2), middle (310mut6) or low (310mutB) secretion efficiency were used in comparison to AMJ627 (SPUSP). As shown, 310mut2 yielded over 150 mg/L Nuc representing a 45% improvement with respect to SP310 (Table 5 below). For 310mut6, the values obtained confirmed a better performance (25% improvement compared to SP310) and 310mutB yielded 50% of the amount secreted using SP310 resembling the results of the initial screening in GM17. Interestingly, the yield of 310mut2 in fermentor is comparable to SPUSP, reaching 85% of the amount of Nuc secreted by the latter.

Table 5. Nuclease secretion in fermentor

Strain (SP)	Nuc (mg/L)	Relative amount (%)
PRA162 (310mut0)	106 ± 1.7	100
PRA164 (310mut2)	154 ± 3.1	145
PRA250 (310mut6)	132 ± 0.2	125
PRA170 (310mutB)	54 ± 3.3	51
AMJ627 (SPUSP)	182 ± x.x	172

Strains were grown in SAIV in a fermentor set at 30°C, pH 5.2.

REFERENCES

Ashbaugh, C.D., Alberti, S. and Wessels, M.R. (1998) Molecular analysis of the capsule gene region of group A *Streptococcus*: the hasAB genes are sufficient for capsule expression. *J Bacteriol* **180**:4955-4959.

Camilli, A., Portnoy, A. and Youngman, P. (1990) Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn*917* derivative that allows direct cloning of DNA flanking transposon insertions. *J Bacteriol* **172**:3738-3744.

Chen, M., and Nagarajan, V. (1994) Effect of alteration of charged residues at the N termini of signal peptides on protein export in *Bacillus subtilis*. *J Bacteriol* **176**:5796-5801.

Chen, H., Kim, J., and Kendall, D.A. (1996) Competition between functional signal peptides demonstrates variation in affinity for the secretion pathway. *J Bacteriol* **178**:6658-6664.

Collier, D.N. (1994) *Escherichia coli* signal peptides direct inefficient secretion of an outer membrane protein (OmpA) and periplasmic proteins (maltose-binding protein, ribos - binding protein, and alkaline phosphatase) in *Bacillus subtilis*. *J Bacteriol* **176**:3013-3020.

Gasson, M. (1983) Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **154**:1-9.

Godon, J.J., Jury, K., Shearman, C.A. and Gasson M.J. (1994) The *Lactococcus lactis* sex-factor aggregation gene *cluA*. *Mol Microbiol* **12**:655-663

Grant, S.G., Jesse, J., Bloom, F.R. and Hanahan, D. (1990) Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad Sci* **USA 87**:4645-4649.

Holo, H., and Nes, I.F. (1989) High-frequency transformation, by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol* **55**:3119-3123.

Israelsen, H. and Hansen, E.B. (1993) Insertion of transposon Tn*917* derivatives into the *Lactococcus lactis* subsp. *lactis* chromosome. *Appl Environ Microbiol* **59:**21-26.

Israelsen, H., Madsen, S.M., Vrang, A., Hansen, E.B., and Johansen, E. (1995) Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn*917-lacZ* integrants with the new promoter probe vector pAK80. *Appl Environ Microbiol* **61:**2540-2547.

Jensen, P.R., and Hammer, K. (1993) Minimal requirements for exponential growth *Lactococcus lactis*. *Appl Environ Microbiol* **59**:4363-4366.

Izard, J.W., Doughty, M.B., Kendall, D.A. (1995) Physical and conformational properties of synthetic idealized signal sequences parallel their biological function. *Biochemistry* **34**:9904-9912.

Izard, J.W., Rusch, S.L., and Kendall, D.A. (1996) The amino-terminal charge and core region hydrophobicity interdependently contribute to the function of signal sequences. *J Biol Chem* **271**:21579-21582.

Lachica, R.V., Genigeorgis, C. and Hoeprich, P.D. (1971) Metachromatic agar-diffusion methods for detecting staphylococcal nuclease activity. *Appl Microbiol* **21**:585-587.

Le Loir, Y., Gruss, A., Ehrlich, S.D., and Langella, P. (1994) Direct screening of recombinants in gram-positive bacteria using the secreted staphylococcal nuclease as a reporter. *J Bacteriol* **176**:5135-5139.

Madsen S.M., Albrechtsen, B., Hansen, E.B. and Israelsen, H. (1996) Cloning and transcriptional analysis of two threonine biosynthetic genes from *Lactococcus lactis* MG1614. *J Bacteriol* **178**:3689-3694

Madsen, S.M., Arnau, J., Vrang, A., Givskov, M., and Israelsen, H. (1999) Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of *Lactococcus lactis*. *Mol Microbiol* **32**:75-87.

Martoglio, B., and Dobberstein, B. (1998) Signal sequences: more than just gr asy peptides. *Trends Cell Biol* **8**:410-415.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int. Neural Sys* **8**:581-599.

Poquet, I., Ehrlich, S.D., and Gruss, A. (1998) An export-specific reporter designed for gram-positive bacteria: application to *Lactococcus lactis*. *J Bacteriol* **180**:1904-1912.

Ravn, P., Arnau, J., Madsen, S., Vrang, A., and Israelsen, H. (1999) The development of Tn*Nuc* and its use for the isolation of novel secretion signals in *Lactococcus lactis*. (in press)

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Santini, C.L., Ize, B., Chanal, A., Muller, M., Giordano, G., Wu, L.F. (1998) A novel sec-independent periplasmic protein translocation pathway in *Escherichia coli*. *EMBO J* **17**:101-112.

Sauve, D.M., Ho, D.T. and Roberge, M. (1995) Concentration of dilute protein for gel electrophoresis. *Anal Biochem* **226**:382-383.

Settles, A.M., and Martienssen, R. (1998) Old and new pathways of protein export in chloroplasts and bacteria. *Trends Cell Biol* **8**:494-501.

Sherrat, D. (1989) Tn3 and related transposable elements: site-specific recombination and transposition. *In*: Berg, E.D. and Howe, M.M. (eds.) Mobile DNA, American Society for Microbiology, Washington DC, USA, pp. 163-184.

Stephens, C. (1998) Protein secretion: getting folded proteins across membranes. *Curr Biol* **8**:578-581.

Takimura, Y., Kato, M., Ohta, T., Yamagata, H., and Udaka, S. (1997) Secretion of human interleukin-2 in biologically active form by *Bacillus brevis* directly into culture medium. *Biosci Biotechnol Biochem* **61**:1858-1861.

van Asseldonk M., Rutten, G., Oteman, M., Siezen, R.J., de Vos, W.M. and Simons G. (1990) Cloning of *usp45*, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. *Gene* **95**:155-60.

van Asseldonk M., de Vos. W.M., and Simons, G. (1993) Functional analysis of the *Lactococcus lactis usp45* secretion signal in the secretion of a homologous proteinase and a heterologous alpha-amylase. *Mol Gen Genet* **240**:428-434.

von Heijne, G. (1990) The signal peptide. *J Mol Biol* **115**:195-201.

Wang, L.F., Kortt, A.A., and Stewart, D.J. (1993) Use of a gram- signal peptide for protein secretion by gram+ hosts: basic protease of *Dichelobacter nodosus* is produced and secreted by *Bacillus subtilis*. *Gene* **131**:97-102.

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A. and Turner, R.J. (1998) A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* **93**:93-101.

Whiting, G.C., Sutcliffe, I.C. and Russell, R.R. (1993) Metabolism of polysaccharides by the *Streptococcus mutants dexB* gene product. *J Gen Microbiol* **139**:2019-2026.

Youngman, P. (1987) Plasmid vectors for recovering and exploiting Tn*917* transpositions in Bacillus and other Gram-positive bacteria. *In*: Hardy, K.G. (ed) Plasmids, a practical approach, IRL Press, Oxford, UK, pp. 79-103.

CLAIMS

1. A method of constructing a transposon derivative for identifying in a lactic acid bacterium a DNA sequence coding for a signal peptide (SP), the method comprising the steps of

(i) selecting a DNA molecule comprising a transposon including between its left terminus (LR) and its right terminus (RR) a sequence comprising a promoterless promoter reporter gene and a ribosome binding site (RBS),

(ii) deleting from said DNA molecule a region located between the LR and the promoterless reporter gene to obtain a modified DNA molecule that has retained its transposability and its RBS,

(iii) inserting into the remaining region located between the LR and the promoterless reporter gene of the resulting modified DNA molecule a unique restriction site, and

(iv) inserting into said unique restriction site a DNA sequence coding for a secretion reporter molecule, said DNA sequence coding for a reporter molecule is without a sequence coding for a SP,

the thus obtained transposon derivative being without stop codons in-frame with the secretion reporter molecule thus permitting upon transposition translational fusions from upstream the LR.

2. A method according to claim 1 wherein the selected DNA molecule comprises the *Tn917* transposon or a derivative hereof.

3. A method according to claim 1 or 2 wherein the promoterless promoter reporter gene is the *lacZ* gene.

4. A method according to any of claims 1-3 wherein the gene coding for the secretion reporter codes for a nuclease.

5. A method according to claim 4 wherein the gene coding for a nuclease is the *nuc* gene derived from *Staphylococcus aureus*.

6. A transposon derivative molecule for the identification in a lactic acid bacterium of a DNA sequence coding for a signal peptide (SP), the molecule comprising the following elements:

(i) a DNA molecule comprising a transposon element including between its left terminus (LR) and its right terminus (RR) a sequence comprising a promoterless promoter reporter gene and a ribosome binding site, the DNA molecule being without stop codons in the region upstream of the promoter reporter gene,

(ii) a DNA sequence coding for a secretion reporter molecule, said DNA sequence is without a sequence coding for an SP.

7. A transposon derivative according to claim 6 where the transposon element is derived from *Tn917* or a derivative hereof.

8. A transposon derivative according to claim 6 or 7 where the promoter reporter gene is the *lacZ* gene.

9. A transposon derivative according to any of claims 6-8 where the DNA sequence coding for a secretion reporter gene is a gene coding for a nuclease.

10. A transposon derivative according to claim 9 where the gene coding for a nuclease is the *nuc* gene derived from *Staphylococcus aureus*.

11. A transposon derivative according to claim 6 which is pTn*Nuc*.

12. A transposon derivative according to claim 6 that further comprises a selection marker.

13. A method of identifying in a lactic acid bacterium a DNA sequence coding for a signal peptide (SP), the method comprising the steps of

(i) transforming a lactic acid bacterium with a transposon derivative according to any of claims 6-12,

(ii) selecting from the transformed lactic acid bacterium, cells in which the promoterless promoter reporter gene is expressed and the gene product of the DNA sequence coding for a secretion reporter molecule is secreted.

14. A method according to claim 13 wherein the lactic acid bacterium being transformed is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc spp.*, *Oenococcus* spp. and *Streptococcus* spp.

15. A method according to claim 13 wherein the transposon derivative is transposed quasi-randomly.

16. A method of isolating from a source lactic acid bacterium a DNA sequence coding for a signal peptide (SP), the method comprising identifying such a sequence using the method of any of claims 13-15, and isolating said sequence.

17. A method according to claim 16 wherein the lactic acid bacterium is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc spp.*, *Oenococcus* spp. and *Streptococcus* spp.

18. An isolated DNA molecule comprising at least part of a transposon derivative according to any of claims 6-12 and a DNA sequence coding for a signal peptide (SP) that is functional in a lactic acid bacterium.

19. A DNA molecule according to claim 18 where the signal peptide comprises a signal peptidase I-recognition sequence.

20. A DNA molecule according to claim 18 where the signal peptide comprises a signal peptidase II-recognition sequence.

21. A DNA molecule according to claim 18 where the DNA sequence coding for a signal peptide is derived from a clone selected from the group consisting of SP10, SP307, SP310 and SP330, and a mutant thereof.

22. A DNA molecule according to claim 21 where the signal peptide is a mutant of the signal peptide derived from SP310.

23. A DNA molecule according to claim 22 where the mutant is selected from the group consisting of 310mut1, 310mut2, 310mut3, 310mut4, 310mut5, 310mut6, 310mut7, 310mut8, 310mut10, 310mut11, 310mutA, 310mutB, 310mutC, 310mutA1, 310mutB1, 310mutD2, 310mutD7, 310mutE2, 310mutE11 and 310mutF2.

24. An isolated DNA sequence coding for a signal peptide that is derived from a molecule selected from the group consisting of SP10, SP13, SP307, SP310 and SP330, and a derivative of any of said signal peptides having retained signal peptide functionality.

25. A DNA sequence according to claim 24 where the signal peptide is a mutant of the signal peptide derived from SP310.

26. A DNA sequence according to claim 25 where the mutant is selected from the group consisting of 310mut1, 310mut2, 310mut3, 310mut4, 310mut5, 310mut6, 310mut7, 310mut8, 310mut10, 310mut11, 310mutA, 310mutB, 310mutC, 310mutA1, 310mutB1, 310mutD2, 310mutD7, 310mutE2, 310mutE11 and 310mutF2.

27. A recombinant plasmid comprising an isolated DNA molecule comprising at least part of a transposon derivative according to any of claims 18-23 or an isolated DNA sequence according to any of claims 24-26.

28. A recombinant plasmid according to claim 27 that is selected from the group consisting of Δ10::Nuc, Δ13::Nuc, Δ307::Nuc and Δ310::Nuc.

29. A recombinant plasmid according to claim 27 or 28 that further comprises a regulatable promoter operably linked to the *nuc* gene.

30. A plasmid according to claim 29 wherein the regulatable promoter is P170.

31. A recombinant bacterium comprising a DNA sequence according to any of claims 24-26.

32. A bacterium according to claim 31 wherein the DNA sequence is operably linked to a gene expressing a desired gene product whereby the gene product is secreted.

33. A bacterium according to claim 31 that is a lactic acid bacterium.

34. Use of a bacterium according to claim 31 for the production of a desired gene product.

35. Use according to claim 34 where the bacterium is a lactic acid bacterium.

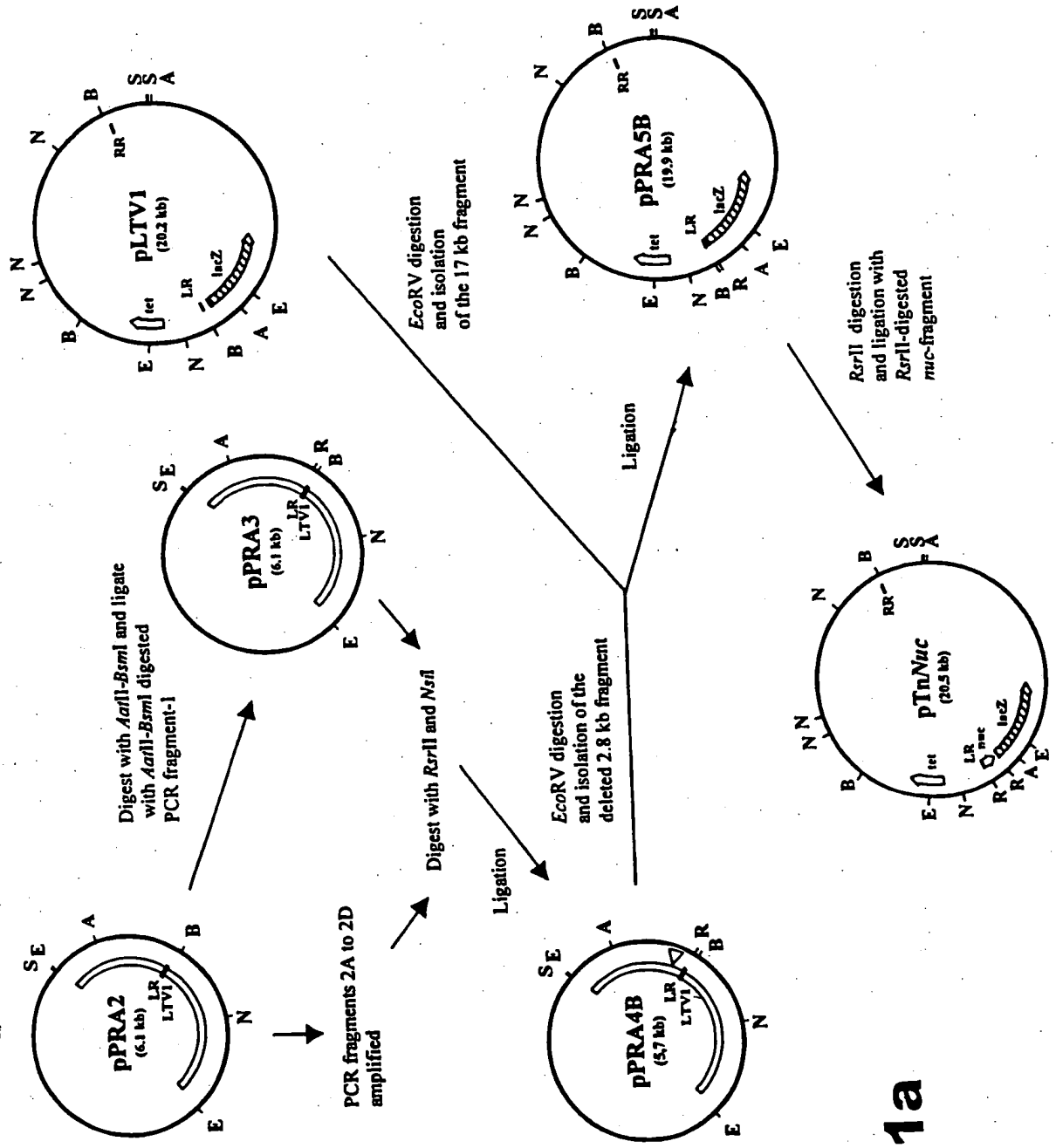
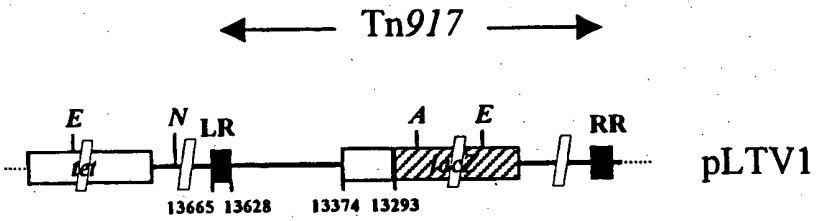

pLTV1
(20.2 kb)
pPRA5B
(19.9 kb)
pPRA3
(6.1 kb)
pPRA2
(6.1 kb)
pPRA4B
(5.7 kb)
pTnNuc
(20.5 kb)
Digest with AarII-BsmI and ligate with AarII-BsmI digested PCR fragment-1
PCR fragments 2A to 2D amplified
Digest with RsrII and NsiI
EcoRV digestion and isolation of the 17 kb fragment
EcoRV digestion and isolation of the deleted 2.8 kb fragment
Ligation
Ligation
RsrII digestion and ligation with RsrII-digested nuc-fragment

Fig. 1a



Plasmid	Deleted Region	Sequence Position	Transposition
pPRA5A	———	13647-13374	-
pPRA5B	———	13626-13374	+
pPRA5C	———	13608-13374	+
pPRA5D	———	13589-13374	+

Fig. 1b

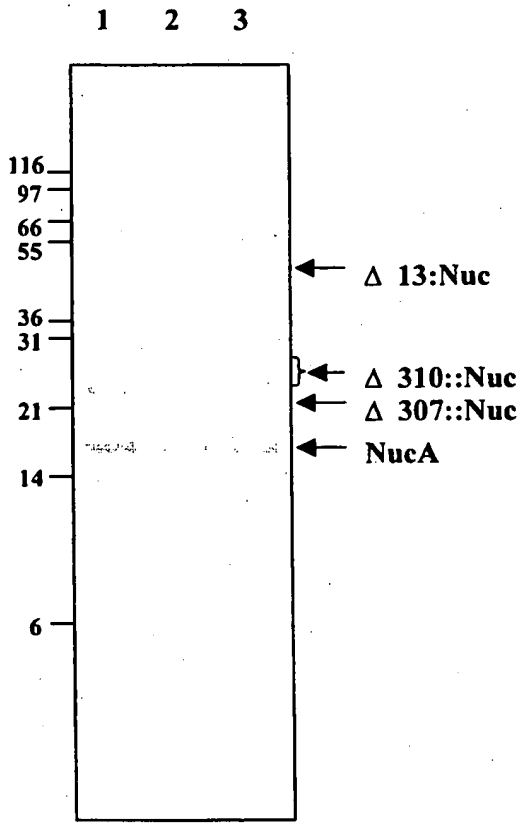


Fig. 2

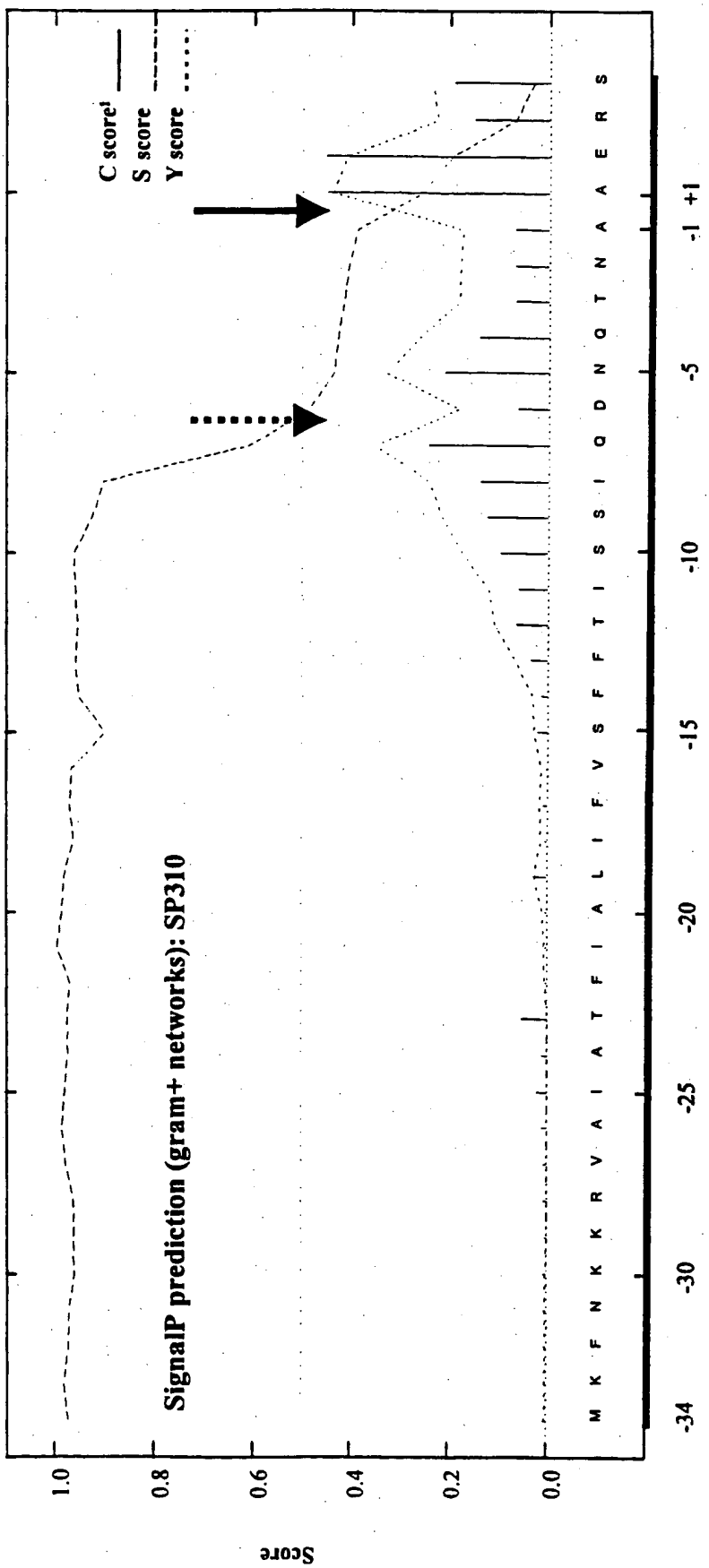

C score
S score
Y score
SignalP prediction (gram+ networks): SP310
M K F N K K R V A I A T F I A L I F V S F F T I S S I Q D N Q T N A A E R S
-34 -30 -25 -20 -15 -10 -5 -1 +1
Position
Score
0.0 0.2 0.4 0.6 0.8 1.0

Fig. 3

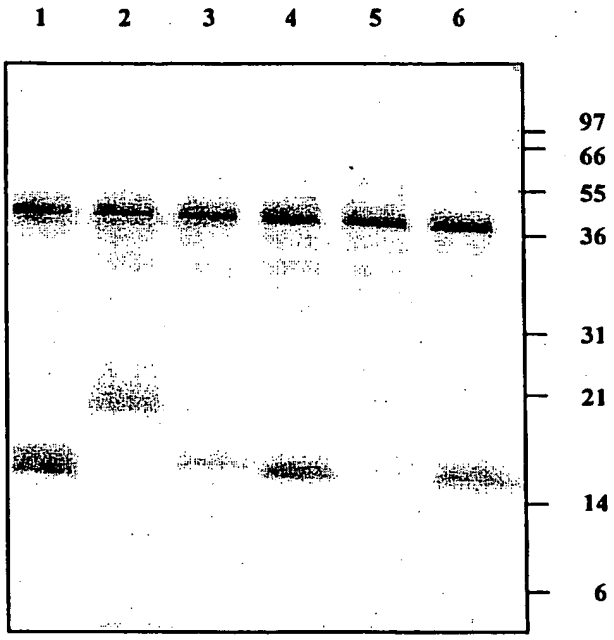


Fig. 4

SEQUENCE LISTING

<110> Bioteknologisk Institut

<120> METHOD OF ISOLATING SECRETION SIGNALS IN LACTIC ACID BACTERIA AND NOVEL SECRETION SIGNALS ISOLATED FROM LACTOCOCCUS LACTIS

<130> 21813PC1

<160> 62

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 1
cgatgaatgc cggaccgaat tgatacacta atgcttttat ataggg 46

<210> 2
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 2
gtgtagtcgg tttatgcagc 20

<210> 3
<211> 19
<212> DNA
<213> Artificial Sequence

<400> 3
cacacatacc aatacatgc 19

<210> 4
<211> 28
<212> DNA
<213> Artificial Sequence

<400> 4
gcatcggtcc gtaggcgctc gggacccc 28

<210> 5
<211> 34
<212> DNA
<213> Artificial Sequence

<400> 5
gcatcggtcc gttcttatcg atacaaattc ctcg 34

<210> 6
<211> 35
<212> DNA
<213> Artificial Sequence

<400> 6
gcatcggtcc gaaattttta aatctatttc ttatc 35

<210> 7
<211> 36
<212> DNA
<213> Artificial Sequence

<400> 7
gcatcggtcc gtaaatgtac aaaataacag cgaaat 36

<210> 8
<211> 30
<212> DNA
<213> Artificial Sequence

<400> 8
gcatcggacc gtcacaaaca gataacggcg 30

<210> 9
<211> 30
<212> DNA
<213> Artificial Sequence

<400> 9
gcatcggtcc gcattattga cctgaatcag 30

<210> 10
<211> 27
<212> DNA
<213> Artificial Sequence

<400> 10
ggaagatctt cacaaacaga taacggc 27

<210> 11
<211> 38
<212> DNA
<213> Artificial Sequence

<400> 11
acgcgtcgac gaattcgatc taaaattata aaagtgcc 38

<210> 12
<211> 75
<212> DNA
<213> Artificial Sequence

<400> 12
gcatcccggg tctagattag ggtaactttg aaaggatatt cctcatgaaa aaaacattga 60
gagaccagtt acttg 75

<210> 13

<211> 39
<212> DNA
<213> Artificial Sequence

<400> 13
gcatagatct actccaacta tcacctgttg catttgctc 39

<210> 14
<211> 76
<212> DNA
<213> Artificial Sequence

<400> 14
gcatcccggg tctagattag ggtaactttg aaaggatatt cctcatgaat aaatcaaaaa 60
ttattgcttt ctctgc 76

<210> 15
<211> 39
<212> DNA
<213> Artificial Sequence

<400> 15
gcatagatct atcaatggaa ttaacatcag ctgccatgc 39

<210> 16
<211> 77
<212> DNA
<213> Artificial Sequence

<400> 16
gcatcccggg tctagattag ggtaactttg aaaggatatt cctcatgaaa tttataaaaa 60
aaagagttgc aatagcc 77

<210> 17
<211> 39
<212> DNA
<213> Artificial Sequence

<400> 17
gcatagatct gttatcatta aaatcactcc gattaagag 39

<210> 18
<211> 72
<212> DNA
<213> Artificial Sequence

<400> 18
tagtaggatc ccgggtctag attagggtaa ctttgaaagg atattcctca tgaaaaaaaa 60
gattatctca gc 72

<210> 19
<211> 40
<212> DNA
<213> Artificial Sequence

<400> 19
acgcgtcgac ctgcagagat cttgtgtcag cgtaaacacc 40

<210> 20

<211> 77
<212> DNA
<213> Artificial Sequence

<400> 20
gcatcccggg tctagattag ggtaactttg aaaggatatt cctcatgaaa tttaataaaa 60
aaagagttgc aatagcc 77

<210> 21
<211> 34
<212> DNA
<213> Artificial Sequence

<400> 21
ctattggttt gattacgtcg gctttctaga tacg 34

<210> 22
<211> 38
<212> DNA
<213> Artificial Sequence

<400> 22
ggttctattg gttcgattac gtcggctttc tagatacg 38

<210> 23
<211> 45
<212> DNA
<213> Artificial Sequence

<400> 23
gttatagtag ttaggttcta cgagttcgtc ggctttctag atacg 45

<210> 24
<211> 62
<212> DNA
<213> Artificial Sequence

<400> 24
ctataaacat tcaaaaaaat gttattaggg tttgtgacga gttcgtcggc tttctagata 60
cg 62

<210> 25
<211> 62
<212> DNA
<213> Artificial Sequence

<400> 25
ctataaacat tcaaaaaaat gttattaggt tttggtttga ttacgtcggc tttctagata 60
cg 62

<210> 26
<211> 62
<212> DNA
<213> Artificial Sequence

<400> 26
ctataaacat tcaaaaaaat gttattaggt tttggttcga ttacgtcggc tttctagata 60
cg 62

<210> 27
<211> 45
<212> DNA
<213> Artificial Sequence

<400> 27
gttatagtag ttaggttcta cgagttcgtc tatgatctag atacg 45

<210> 28
<211> 42
<212> DNA
<213> Artificial Sequence

<400> 28
gttatagtag ttagctacga gttcgtcggc tttctagata cg 42

<210> 29
<211> 42
<212> DNA
<213> Artificial Sequence

<400> 29
gttatagtag ttaggttcga gttcgtcggc tttctagata cg 42

<210> 30
<211> 88
<212> DNA
<213> Artificial Sequence

<400> 30
cgttatcggt gcaaataaaa aaactataaa cattcaaaaa aatgttatag tagttaggtt 60
ctacgagttc gtcggctttc tagatacg 88

<210> 31
<211> 88
<212> DNA
<213> Artificial Sequence

<400> 31
cgttatcggt gcaaataaaa aaactataaa cataaaaaaa aatgttatag tagttaggtt 60
ctacgagttc gtcggctttc tagatacg 88

<210> 32
<211> 120
<212> DNA
<213> Artificial Sequence

<400> 32
cctcccgggt ctagattagg gtaactttga aaggatattc ctcatgaaat ttaataaaaa 60
aagagttgca atagccttgt ttattgcttt gatatttgta ctttttttc ttatatcatc 120

<210> 33
<211> 120
<212> DNA
<213> Artificial Sequence

<400> 33
cctcccgggt ctagattagg gtaactttga aaggatattc ctcatgaaat ttaataaaaa 60
aagagttctt atacttttgt ttattctttt gatatttgta ctttttttc ttatatcatc 120

<210> 34
<211> 103
<212> DNA
<213> Artificial Sequence

<400> 34
cctcccgggt ctagattagg gtaactttga aaggatattc ctcatgaaat ttaataaaaa 60
aagacttttg cttttgcttt tgcttttgct tttacttctt ttg 103

<210> 35
<211> 73
<212> DNA
<213> Artificial Sequence

<400> 35
gaaaatgaag aaaacgaaaa cgaatatagt agttaggttc tattggtttg attacgtcgg 60
ctttctagat acg 73

<210> 36
<211> 38
<212> DNA
<213> Artificial Sequence

<400> 36
ggttctattg gttcgattac gtcggctttc tagatacg 38

<210> 37
<211> 120
<212> DNA
<213> Artificial Sequence

<400> 37
cctcccgggt ctagattagg gtaactttga aaggatattc ctcatgaaat ttaataaaaa 60
aagagttctt atacttttgt ttattctttt gatatttgta ctttttttc ttatatcatc 120

<210> 38
<211> 74
<212> DNA
<213> Artificial Sequence

<400> 38
gcatcccggg tctagattag ggtaactttg aaaggatatt cctcatgaaa aataaaaaaa 60
gagttgcaat agcc 74

<210> 39
<211> 74
<212> DNA
<213> Artificial Sequence

<400> 39
gcatcccggg tctagattag ggtaactttg aaaggatatt cctcatgaaa tttaaaaaaa 60
gagttgcaat agcc 74

<210> 40
<211> 77
<212> DNA
<213> Artificial Sequence

<400> 40
gcatcccggg tctagattag ggtaactttg aaaggatatt cctcatgaaa tttaaaaaaa 60
aaagagttgc aatagcc 77

<210> 41
<211> 38
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide

<400> 41
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Asn Gln Thr
20 25 30
Asn Ala Ala Glu Arg Ser
35

<210> 42
<211> 38
<212> PRT
<213> Artificial Sequence

<400> 42
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Asn Gln Ala
20 25 30
Asn Ala Ala Glu Arg Ser
35

<210> 43
<211> 36
<212> PRT
<213> Artificial Sequence

<400> 43
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala
20 25 30
Ala Glu Arg Ser
35

<210> 44
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 44
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ile Pro Asn Thr Ala Gln Ala Ala
20 25 30
Glu Arg Ser
35

<210> 45
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 45
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ile Gln Asn Gln Thr Asn Ala Ala
20 25 30
Glu Arg Ser
35

<210> 46
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 46
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ile Gln Asn Gln Ala Asn Ala Ala
20 25 30
Glu Arg Ser
35

<210> 47
<211> 36
<212> PRT
<213> Artificial Sequence

<400> 47
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala
20 25 30
Asp Thr Arg Ser
35

<210> 48
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 48
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Asp Ala Gln Ala Ala
20 25 30
Glu Arg Ser
35

<210> 49
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 49
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu

```
 1               5                  10                  15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Ala Gln Ala Ala
            20                  25                  30
Glu Arg Ser
        35

      <210> 50
      <211> 36
      <212> PRT
      <213> Artificial Sequence

      <400> 50
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
 1               5                  10                  15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala
            20                  25                  30
Ala Glu Gly Ser
        35

      <210> 51
      <211> 36
      <212> PRT
      <213> Artificial Sequence

      <400> 51
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Phe Leu
 1               5                  10                  15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala
            20                  25                  30
Ala Glu Arg Ser
        35

      <210> 52
      <211> 36
      <212> PRT
      <213> Artificial Sequence

      <400> 52
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Phe Leu
 1               5                  10                  15
Ile Phe Val Phe Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala
            20                  25                  30
Ala Glu Arg Ser
        35

      <210> 53
      <211> 38
      <212> PRT
      <213> Artificial Sequence

      <220>
      <223> Synthetic signal peptide

      <400> 53
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Leu Phe Ile Ala Leu
 1               5                  10                  15
Ile Phe Val Leu Phe Phe Leu Ile Ser Ser Ile Gln Asp Asn Gln Thr
            20                  25                  30
Asn Ala Ala Glu Arg Ser
```

35

<210> 54
<211> 38
<212> PRT
<213> Artificial Sequence

<400> 54
Met Lys Phe Asn Lys Lys Arg Val Leu Ile Leu Leu Phe Ile Leu Leu
1 5 10 15
Ile Phe Val Leu Phe Phe Leu Ile Ser Ser Ile Gln Asp Asn Gln Thr
20 25 30
Asn Ala Ala Glu Arg Ser
35

<210> 55
<211> 38
<212> PRT
<213> Artificial Sequence

<400> 55
Met Lys Phe Asn Lys Lys Arg Leu Leu Leu Leu Leu Leu Leu Leu Leu
1 5 10 15
Leu Leu Leu Leu Leu Leu Leu Ile Ser Ser Ile Gln Asp Asn Gln Thr
20 25 30
Asn Ala Ala Glu Arg Ser
35

<210> 56
<211> 38
<212> PRT
<213> Artificial Sequence

<400> 56
Met Lys Phe Asn Lys Lys Arg Val Ala Ile Ala Leu Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Leu Phe Phe Leu Ile Ser Ser Ile Gln Asp Asn Gln Ala
20 25 30
Asn Ala Ala Glu Arg Ser
35

<210> 57
<211> 38
<212> PRT
<213> Artificial Sequence

<400> 57
Met Lys Phe Asn Lys Lys Arg Val Leu Ile Leu Leu Phe Ile Leu Leu
1 5 10 15
Ile Phe Val Leu Phe Phe Leu Ile Ser Ser Ile Gln Asp Asn Gln Ala
20 25 30
Asn Ala Ala Glu Arg Ser
35

<210> 58
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 58
Met Lys Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu Ile
1 5 10 15
Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala Ala
20 25 30
Glu Arg Ser
35

<210> 59
<211> 34
<212> PRT
<213> Artificial Sequence

<400> 59
Met Lys Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu Ile
1 5 10 15
Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Asp Ala Gln Ala Ala Glu
20 25 30
Arg Ser

<210> 60
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 60
Met Lys Phe Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu Ile
1 5 10 15
Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala Ala
20 25 30
Glu Arg Ser
35

<210> 61
<211> 35
<212> PRT
<213> Artificial Sequence

<400> 61
Met Lys Asn Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Phe Leu Ile
1 5 10 15
Phe Val Phe Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala Ala
20 25 30
Glu Arg Ser
35

<210> 62
<211> 36
<212> PRT
<213> Artificial Sequence

<400> 62
Met Lys Phe Lys Lys Lys Arg Val Ala Ile Ala Thr Phe Ile Ala Leu
1 5 10 15
Ile Phe Val Ser Phe Phe Thr Ile Ser Ser Ile Gln Asp Ala Gln Ala
20 25 30
Ala Glu Arg Ser
35

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 February 2001 (15.02.2001)

PCT

(10) International Publication Number
WO 01/11060 A3

(51) International Patent Classification[7]: C12N 15/74, 15/62

(21) International Application Number: PCT/DK00/00437

(22) International Filing Date: 4 August 2000 (04.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 1999 01105 6 August 1999 (06.08.1999) DK

(71) Applicant *(for all designated States except US)*: **BIOTEKNOLOGISK INSTITUT** [DK/DK]; Kogle Allé 2, DK-2970 Hoersholm (DK).

(72) Inventors; and

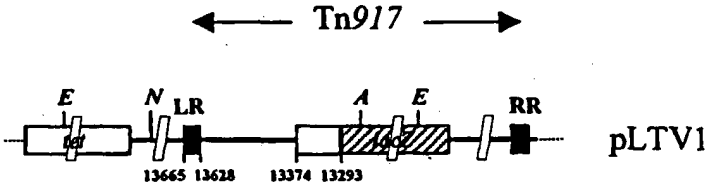
(75) Inventors/Applicants *(for US only)*: **RAVN, Peter** [DK/DK]; Hegnsvej 123, DK-2850 Naerum (DK). **MADSEN, Soeren, Michael** [DK/DK]; 1st floor, Baldersgade 11, DK-2200 Copenhagen N (DK). **VRANG, Astrid** [DK/DK]; Langs Hegnet 76, DK-2800 Lyngby (DK). **ISRAELSEN, Hans** [DK/DK]; Proevestensvej 1A, DK-3450 Alleroed (DK). **JOHNSEN, Mads, Groenvold** [DK/DK]; Sveasvej 5, 3rd floor, DK-1917 Frederiksberg C (DK). **BREDMOSE, Lars** [DK/DK]; Brynhildegade 9, 3rd floor, DK-2200 Copenhagen N (DK). **ARNAU, José** [ES/DK]; Melvillevej 9, DK-2900 Hellerup (DK).

(74) Agent: **PLOUGMANN, VINGTOFT & PARTNERS A/S**; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG,

[Continued on next page]

(54) Title: METHOD OF ISOLATING SECRETION SIGNALS IN LACTIC ACID BACTERIA AND NOVEL SECRETION SIGNALS ISOLATED FROM *LACTOCOCCUS LACTIS*



Plasmid	Deleted Region	Sequence Position	Transposition
pPRA5A	———	13647-13374	-
pPRA5B	———	13626-13374	+
pPRA5C	———	13608-13374	+
pPRA5D	—	13589-13374	+

(57) Abstract: A method of identifying nucleotide sequences coding for signal peptides in lactic acid bacteria, using a DNA molecule comprising a transposon including a promoterless reporter gene from which DNA molecule a region between the LR and the reporter gene is deleted and the DNA molecule comprises a DNA sequence coding for a secretion reporter molecule. By deleting the region between the LR and the reporter gene, stop codons in-frame with the secretion reporter molecule is removed which upon transposition permits translational fusions from upstream the LR.

WO 01/11060 A3

MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
23 August 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/DK 00/00437

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/74 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 914 025 A (MANOIL COLIN ET AL) 3 April 1990 (1990-04-03) the whole document column 6, line 66,67 ---	1-17
X	WO 94 16086 A (BIOTEKNOLOGISK INST ;HANSENS LAB (DK); JOHANSEN ERIC (DK); NILSSON) 21 July 1994 (1994-07-21)	27,28
Y	the whole document page 5, line 5 -page 8, line 24 page 10, line 8-12 examples 1-8 --- -/--	1-3,6-8, 11-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 2001	23.03.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/DK 00/00437

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POQUET I ET AL.: "An export-specific reporter designed for gram-positive bacteria: application to Lactococcus lactis" JOURNAL OF BACTERIOLOGY, vol. 180, no. 7, April 1998 (1998-04), pages 1904-1912, XP002135834 cited in the application	18-23, 27,28
Y	abstract	4,5,9-11

X	ISRAELSEN H ET AL.: "Cloning and partial characterization of regulated promoters from Lactococcus lactis Tn917-lacZ integrants with the new promoter probe vector, pAK80." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, no. 7, July 1995 (1995-07), pages 1540-2547, XP002135836 cited in the application abstract tables 1,2	18, 21-23, 27,28

A	MANOIL C ET AL.: "TnphoA: a transposon probe for protein export signals" PROC. NATL. ACAD. SCI. USA, vol. 82, no. 23, December 1985 (1985-12), pages 8129-8133, XP002135835 abstract	1,6,12, 13,16

A	TADAYYON M ET AL.: "TnblaM: a transposon for directly tagging bacterial genes encoding cell envelope and secreted proteins" GENE, vol. 111, January 1992 (1992-01), pages 21-26, XP000605471 abstract	1,6,12, 13,16

A	ISRAELSEN H ET AL.: "Insertion of transposon Tn917 derivatives into the Lactococcus lactis subsp. lactis chromosome" APPL. ENVIRON. MICROBIOL., vol. 59, no. 1, 1993, pages 21-26, XP000891981 cited in the application abstract	1,2,6,7

	-/--	

4

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LE LOIR Y ET AL.: "Direct screening of recombinants in gram-positive bacteria using the secreted staphylococcal nuclease as a reporter" JOURNAL OF BACTERIOLOGY, vol. 176, no. 16, August 1994 (1994-08), pages 5135-5139, XP000857662 cited in the application abstract	4,5,9,10
A	LANGELLA P ET AL.: "Heterologous protein secretion in Lactococcus lactis: a novel antigen delivery system" BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH, vol. 32, no. 2, February 1999 (1999-02), pages 191-198, XP000857556 abstract	4,5,9,10
A	WO 92 04451 A (GENESIT OY ;VALIO FINNISH CO OPERATIVE DAI (FI)) 19 March 1992 (1992-03-19) page 5, line 23 -page 8, line 16 examples 5-7	1,6,13, 14, 16-20,27
A	SIBAKOV M ET AL.: "Secretion of TEM beta-lactamase with signal sequences isolated from the chromosome of Lactococcus lactis subsp. lactis" APPL. ENVIRON. MICROBIOL., vol. 57, no. 2, February 1991 (1991-02), pages 341-348, XP000891982 cited in the application abstract	1,6,13, 14, 16-20,27
A	PEREZ-MARTINEZ G ET AL: "Protein export elements from Lactococcus lactis." MOLECULAR AND GENERAL GENETICS, vol. 234, no. 3, September 1992 (1992-09), pages 401-411, XP002135837 cited in the application abstract	1,6,13, 14, 16-19,27
A	SMITH H ET AL.: "Construction and use of signal sequence selection vectors in Escherichia coli and Bacillus subtilis" JOURNAL OF BACTERIOLOGY, vol. 169, no. 7, 1 July 1987 (1987-07-01), pages 3321-3328, XP000610632 abstract	1

-/--

4

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/DK 00/00437

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SMITH H ET AL.: "Characterization of signal-sequence-coding regions selected from the Bacillus subtilis chromosome" GENE, vol. 70, no. 2, 30 October 1988 (1988-10-30), pages 351-361, XP000000377 abstract	1
A	WO 96 41891 A (MAX PLANCK GESELLSCHAFT ;HAAS RAINER (DE); ODENBREIT STEFAN (DE);) 27 December 1996 (1996-12-27) page 9, line 7-25 page 11, line 4-28	1,6,13
T	RAVN P ET AL.: "The development of TnNuc and its use for the isolation of novel secretion signals in Lactococcus lactis." GENE, vol. 242, no. 1-2, 25 January 2000 (2000-01-25), pages 347-356, XP002135839 cited in the application the whole document	1-23, 27-30
T	WO 00 06736 A (HANNIFFY SEAN BOSCO ;LE PAGE RICHARD WILLIAM FALLA (GB); WELLS JER) 10 February 2000 (2000-02-10) page 10, line 23 -page 13, line 5 example 1 claims 19,20	4,5,9, 10,18,27

4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00437

Box I Observations where c rtain claims w re found uns archable (Continuation f item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Claims 1-20, (all completely), 21-23, 27-30 (all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20 (all completely); 21-23, 27-30 (all partially)

A method of constructing a transposon derivative for identifying in a lactic acid bacterium (LAB) a DNA sequence coding for a signal peptide (SP), the method comprising the steps of selecting a DNA molecule comprising a transposon (preferably Tn917) comprising between its left and right termini a promoterless promoter reporter gene (preferably lacZ) and a ribosome binding site (RBS), deleting from said DNA molecule a DNA region between the left terminus and the reporter gene in such a way that the transposability and the RBS are retained, inserting into the remaining region between the left terminus and the reporter gene a unique restriction site, and inserting into said restriction site a DNA sequence coding for a secretion reporter molecule (preferably Staphylococcus aureus nuclease) in such a way that the transposon derivative is without stop codons in-frame with the secretion reporter molecule. Also claimed is a transposon derivative as said, methods of identifying and isolating SPs in/from a LAB using a transposon derivative as said, an isolated DNA molecule comprising at least part of a transposon deriviative as said, and a recombinant plasmid comprising an isolated DNA molecule as said.

2. Claims: 21, 24, 27-35 (all partially)

An isolated DNA sequence coding for signal peptide SP10 and a derivative of said SP having retained SP functionality, an isolated DNA molecule or a recombinant plasmid comprising said signal peptide, and a recombinant bacterium, e.g. an lactic acid bacterium, comprising a DNA sequence as said, the DNA sequence preferably being operably linked to a gene expressing a desired gene product so that the gene product is secreted. Also claimed is the use of a bacterium as said for the production of a desired gene product.

3. Claims: 24, 27-35 (all partially)

An isolated DNA sequence coding for signal peptide SP13 and a derivative of said SP having retained SP functionality, a recombinant plasmid comprising said signal peptide, and a recombinant bacterium, e.g. an lactic acid bacterium, comprising a DNA sequence as said, the DNA sequence preferably being operably linked to a gene expressing a desired gene product so that the gene product is secreted. Also claimed is the use of a bacterium as said for the production of a desired gene product.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

4. Claims: 21, 24, 27-35 (all partially)

An isolated DNA sequence coding for signal peptide SP307 and a derivative of said SP having retained SP functionality, an isolated DNA molecule or a recombinant plasmid comprising said signal peptide, and a recombinant bacterium, e.g. an lactic acid bacterium, comprising a DNA sequence as said, the DNA sequence preferably being operably linked to a gene expressing a desired gene product so that the gene product is secreted. Also claimed is the use of a bacterium as said for the production of a desired gene product.

5. Claims: 21-24, 27-35 (all partially); 25, 26 (both completely)

An isolated DNA sequence coding for signal peptide SP310 and a derivative or mutant of said SP having retained SP functionality, an isolated DNA molecule or a recombinant plasmid comprising said signal peptide, and a recombinant bacterium, e.g. an lactic acid bacterium, comprising a DNA sequence as said, the DNA sequence preferably being operably linked to a gene expressing a desired gene product so that the gene product is secreted. Also claimed is the use of a bacterium as said for the production of a desired gene product.

6. Claims: 21, 24, 27, 29, 30, 31-35 (all partially)

An isolated DNA sequence coding for signal peptide SP330 and a derivative of said SP having retained SP functionality, an isolated DNA molecule or a recombinant plasmid comprising said signal peptide, and a recombinant bacterium, e.g. an lactic acid bacterium, comprising a DNA sequence as said, the DNA sequence preferably being operably linked to a gene expressing a desired gene product so that the gene product is secreted. Also claimed is the use of a bacterium as said for the production of a desired gene product.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter nal Application No
PCT/DK 00/00437

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4914025 A	03-04-1990	NONE	
WO 9416086 A	21-07-1994	AU 675821 B	20-02-1997
		AU 5832594 A	15-08-1994
		CA 2152898 A	21-07-1994
		EP 0677110 A	18-10-1995
		JP 8500739 T	30-01-1996
		NZ 259510 A	24-06-1997
		NZ 286635 A	24-06-1997
		US 5837509 A	17-11-1998
WO 9204451 A	19-03-1992	AU 652920 B	15-09-1994
		EP 0550428 A	14-07-1993
		FI 930832 A	28-04-1993
		JP 6503945 T	12-05-1994
WO 9641891 A	27-12-1996	DE 19521312 A	19-12-1996
		AU 707741 B	15-07-1999
		AU 6126396 A	09-01-1997
		CA 2224567 A	27-12-1996
		EP 0833943 A	08-04-1998
WO 0006736 A	10-02-2000	NONE	